Universidade de Lisboa
Faculdade de Medicina da Universidade de Lisboa

Topical bacteriophage therapy of the infected diabetic foot

Doutoramento em Medicina
Especialidade de Medicina Interna

João João Mendes
2014

Universidade de Lisboa
Faculdade de Medicina da Universidade de Lisboa

Topical bacteriophage therapy of the infected diabetic foot

Tese orientada pelo Professor Doutor José Melo Cristino da Faculdade de Medicina da Universidade de Lisboa, e co-orientada pela Professora Doutora Patrícia Cavaco Silva do Instituto Superior de Ciências da Saúde Egas Moniz e pelo Professor Doutor Andrzej Górski da The Medical University of Warsaw / Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw

Doutoramento em Medicina
Especialidade de Medicina Interna

João João Mendes
2014

A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina da Universidade de Lisboa em reunião de 22 de julho de 2014

Table of Contents

Abstract

Diabetes mellitus is a global epidemic, with an estimated 171 million diabetic patients worldwide. Diabetic wound infections, particularly diabetic foot infections (DFI), are a major source of morbidity and their treatment consumes a significant amount of medical economic resources. In addition to debridement, the current first-line treatment for DFIs is antibiotic therapy. However, an increasing number of antibiotic-resistant DFI cases require alternative treatments. One potential alternative is bacteriophage therapy (BT). The purpose of this study is to develop and evaluate a topically-delivered bacteriophage treatment protocol (including formulation and dosage regimen) with potential efficacy in diabetic wound infections.

The principles of wound infection management are discussed, and a comprehensive overview of DFI assessment and management is provided. The principles and current applications of BT are also examined, with particular regard to the potential for the inclusion of topical BT in the DFI therapeutic strategy.

In order to provide a thorough understanding of the epidemiology of DFI, a transversal observational multicenter study was conducted. Forty-nine hospitalized and ambulatory patients were enrolled, and 147 microbial isolates, comprising 43 different species, were cultured. This survey enabled the identification, characterization, and clinical correlation of the main bacteria involved, thus allowing the choice of the bacterial targets (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*). These targets were chosen not only because of their frequency but also because of their association with multi-drug resistance.

Following the epidemiological study, five previously morphologically and genetically characterized bacteria-specific lytic bacteriophages (*S. aureus* F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 bacteriophages) were tested against these strains. The bacteriophage spot test procedure was used, and their combination was shown to have broad host ranges for the different target bacterial species. Subsequently, the in vitro activity against planktonic cells and established biofilms was studied. Target bacteria during planktonic growth were challenged with their specific bacteriophages, which revealed effective, early killing at 4 hours, but this was followed by bacterial regrowth to pre-treatment levels within 24 hours. However, by using metabolic activity as a measure of cell viability within established biofilms, significant cell impairment following bacteriophage exposure was found when repeated treatment (every 4 hours) was applied. The greatest effects occurred in both planktonic and biofilm cells, showing a bacteriophage/bacterium input multiplicity (IM) of 10. These complementary studies on both planktonic cells and established biofilms allowed us to define a high IM (≥10) and a treatment protocol of multiple doses (every 4 hours for 24 hours).

When in vitro testing showed the feasibility of the project and indicated the optimal dosage regimen, the opportunity to study the bacteriophage solutions led to the development of an animal model of diabetic wound infection, which was suitable for the evaluation of topical antimicrobial therapies. The Galiano et al. murine splinted, excisional wound-healing model was used as a template and further refined and adapted using male chemically induced diabetic Wistar rats. After every step of the procedure was optimized, a model was established, which accurately reproduced not only the healing of infected wounds in humans but also the current treatment standard of care, including sharp debridement.

This led to the subsequent in vivo testing of the antimicrobial activity and wound-healing capability of the topically delivered bacteriophage protocol against wounds with *S. aureus*, *P. aeruginosa*, and

A. baumannii infections, using single strains of the bacteria, the aforementioned animal model, and a previously optimized pig wound infection model. In conjunction with sharp debridement, the bacteriophage treatment effectively decreased bacterial colony counts and improved planimetric and histological parameters in *S. aureus* and *P. aeruginosa* infections, but was not as effective against *A. baumannii*. Although the improvements were more significant in the rodent model than in the porcine model, our results suggested that topically administered bacteriophage treatment when applied in conjunction with wound debridement may be effective in resolving chronic wound infections. Incidentally, the results of the in vitro and in vivo studies were not only complementary but also yielded similar microbiological results, further highlighting the consistency and efficacy of the protocol across the tested scenarios.

However, this project is not without limitations. Only one strain each of the bacteria was used in the in vivo testing, which meant that the effectiveness of the treatment was limited to these strains and cannot be universally assumed to be effective against related, similar, or other untested strains. Moreover, although the animal models are appropriate in terms of their biological similarity to humans, they fail to account for the full complexity of human DFIs, which could influence the effectiveness of the protocol. Furthermore, only one type of BT protocol was tested. It was demonstrably effective, but it may not be the most efficacious form of BT protocol. Additionally, bacteriophage resistance was not examined or tested, so even if the BT protocol is effective, this study cannot predict outcomes for all scenarios in which the BT protocol may be used.

Nevertheless, collectively these studies show that a high IM (≥10) and multiple dose (every 4 hours for 24 hours), topical BT protocol may be an effective therapeutic approach to diabetic wounds infected with different pathogens, particularly those identified as antibiotic-resistant. These results represent the first step in the development of a fully regulated human clinical trial that explores the potential of BT in diabetic wound infections. The ultimate goal of this study is to transform BT into a viable, everyday strategy for treating DFIs.

Resumo

A diabetes *mellitus* configura-se hoje como uma epidemia global, com uma estimativa mundial de cerca de 171 milhões de diabéticos. As infeções de feridas em diabéticos, particularmente as infeções do pé diabético, são uma importante fonte de morbidade, e representam uma significativa fonte de consumo de recursos médicos e económicos. A par do desbridamento mecânico, o atual tratamento de primeira linha para as infeções do pé diabético é a terapêutica antibiótica. No entanto, são cada vez mais as infeções por bactérias resistentes aos antibióticos, o que incita ao desenvolvimento de terapêuticas alternativas. Uma alternativa potencial é a terapêutica bacteriofágica. O objetivo deste estudo foi o desenvolvimento e avaliação de um protocolo de terapêutica bacteriofágica tópica (incluindo a formulação e posologia) com potencial eficácia no tratamento de infeções de feridas de diabéticos.

São abordados os princípios gerais da terapêutica das infeções da pele e tecidos moles, com especial referência às infeções do pé diabético. Também os princípios gerais e as aplicações atuais e potenciais da terapêutica bacteriofágica são detalhados, destacando-se o potencial para a sua inclusão na estratégia terapêutica nas infeções do pé diabético.

A fim de proporcionar uma visão global da epidemiologia das infeções do pé diabético, conduziu-se um estudo observacional, multicêntrico e transversal. Foram recrutados 49 doentes de ambulatório e em hospitalização, tendo-se isolado 147 microrganismos, pertencendo a 43 diferentes espécies. Este estudo permitiu a identificação, caracterização e correlação clínica das principais bactérias responsáveis pelas infeções do pé diabético, permitindo, assim, a escolha dos alvos bacterianos (*Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Acinetobacter baumannii*). Estes microrganismos foram escolhidos não só pela sua frequência, mas também pela sua associação à resistência aos antibióticos.

Na sequência do estudo epidemiológico, foram testados nestas estirpes cinco bacteriófagos líticos bactéria-específicos previamente caracterizados morfológica e geneticamente (bacteriófagos *S. aureus* F44/10 e F125/10, *P. aeruginosa* F770/05 e F510/08 e *A. baumannii* F1245/05), utilizando o procedimento de *spot test* para bacteriófagos. As combinações de bacteriófagos bactéria-específicos demostraram ter amplos *host range* para as diferentes espécies de bactérias-alvo. Posteriormente, a atividade destas combinações sobre células planctónicas e em biofilme maduro foi estudada *in vitro*. As bactérias-alvo em crescimento planctónico foram infetadas com diferentes combinações dos seus bacteriófagos específicos, obtendo-se eficácia inicial (nas primeiras 4 horas), porém, com recrescimento bacteriano para os níveis pré-tratamento em 24 horas. No entanto, em células de biofilmes maduros – usando a atividade metabólica como uma medida da viabilidade celular – obteve-se uma importante limitação da viabilidade após exposição repetida (a cada 4 horas) às diferentes combinações dos bacteriófagos específicos. Os maiores efeitos, tanto sobre células planctónicas como sobre as células em biofilme, ocorreu com uma *input multiplicity* de 10. Estes estudos complementares em células planctónicas e em biofilme maduro permitiram definir um protocolo terapêutico utilizando uma *input multiplicity* elevada (≥ 10) e múltiplas doses (a cada 4 horas durante 24 horas).

Uma vez que os testes *in vitro* mostraram a viabilidade do projeto e indicaram a posologia ideal, a oportunidade de estudar as soluções de bacteriófagos levou ao desenvolvimento de um modelo animal de infeção da ferida diabética adequado para a avaliação das terapêuticas tópicas antimicrobianas. O modelo murino excisional de cicatrização de feridas com utilização de tala de Galiano *et al.* foi adaptado e aperfeiçoado, utilizando ratos Wistar machos com diabetes *mellitus*

induzida quimicamente. Depois de otimizar cada etapa do processo, foi estabelecido um modelo que não só reproduz com precisão o processo de cicatrização de feridas infetadas em seres humanos, mas que reproduz igualmente o atual protocolo de tratamento destas feridas, incluindo o desbridamento mecânico.

Isto levou ao subsequente ensaio *in vivo* – no modelo animal acima mencionado, bem como num outro modelo porcino de infeção de ferida previamente otimizado – da atividade antimicrobiana e da capacidade de cicatrização de feridas do protocolo de terapêutica bacteriofágica tópica em feridas infetadas por estirpes individuais de *S. aureus*, *P. aeruginosa* e *A. baumannii*. A terapêutica bacteriofágica tópica, em conjunto com o desbridamento mecânico, resultou efetivamente na diminuição da contagem de colónias bacterianas bem como na redução da área de ferida e melhoria dos parâmetros histológicos em feridas infetadas por *S. aureus* e *P. aeruginosa*, não tendo sido demonstrada tanta eficácia nas infetadas por *A. baumannii*. Estes resultados, embora mais expressivos no modelo de roedor do que no modelo porcino, sugerem que a terapêutica bacteriofágica tópica, quando aplicada em conjunto com o desbridamento mecânico, pode ser eficaz na resolução de infeções de feridas crónicas. Aliás, os resultados dos ensaios *in vitro* e *in vivo* não só são complementares, como também apresentam resultados microbiológicos semelhantes, destacando a consistência e eficácia do protocolo entre os cenários testados.

No entanto, este projeto não é isento de limitações. Apenas uma estirpe de cada uma das bactérias foi usada no teste *in vivo*, o que significa que a evidência da eficácia terapêutica é limitada a estas estirpes, não podendo ser assumida como eficácia universal, nomeadamente em estirpes não testadas. Ademais, e independentemente da similaridade biológica dos modelos animais com os humanos, eles não conseguem dar conta de toda a complexidade das infeções do pé diabético que pode influenciar a eficácia do protocolo. Além disso, apenas um tipo de protocolo de terapêutica bacteriofágica foi testado. Apesar de ter demonstrado eficácia, pode não ser o protocolo mais eficaz. Por fim, também a resistência aos bacteriófagos não foi avaliada, o que significa que, mesmo que o protocolo seja eficaz, o estudo não pode prever os resultados para todos os cenários em que o protocolo possa ser utilizado.

Coletivamente, estes estudos demostram que um protocolo de terapêutica bacteriofágica tópica usando uma *input multiplicity* alta (≥ 10) e doses múltiplas (a cada 4 horas durante 24 horas) pode ser uma abordagem terapêutica eficaz para a terapêutica de feridas diabéticas infetadas por diferentes agentes patogénicos, especialmente aqueles associados a resistência aos antibióticos. Estes resultados representam um primeiro passo no desenvolvimento de um ensaio clínico controlado e regulamentado para a avaliação do potencial da terapêutica bacteriofágica nas infeções de feridas de diabéticos. O objetivo final deste estudo é transformar a terapêutica bacteriofágica numa alternativa viável para o tratamento das infeções do pé diabético.

– to the memory of Professor Cristina Vilela†,
who never stopped believing

– to my parents,
who laid the foundations of the man I am

– to my grandparents,
who gave me the love and time that made everything else possible

– to Maria Maria,
who taught me that life is not just Medicine

– to Natália, João, and António,
who give meaning to everything I do

Acknowledgements

The path towards completing this thesis has involved several years of work, and many people have contributed to the ideas presented and the knowledge gained. The author recognizes his debt to those who helped and positively influenced him along the way. It is, however, not possible to list them all here.

First, I want to thank my primary supervisor, Professor Melo Cristino, who gave me the latitude to explore the topic in my own way while constantly providing encouragement, advice, and guidance. I also wish to express my gratitude to my co-supervisors, Professor Patrícia Cavaco Silva and Professor Andrzej Górski, for their continuous encouragement and invaluable suggestions during this work.

I gratefully acknowledge the funding sources that made the work for my doctoral thesis possible. I would like to thank Professor Miguel Garcia, who enabled this research work at Technophage—Investigação e Desenvolvimento em Biotecnologia, S.A. and, together with Tecnifar—Indústria Técnica Farmacêutica, S.A., provided funding for this research. My work was also supported by a Fundação para a Ciência e a Tecnologia PhD studentship grant for medical residents (SFRH/SINTD/60025/2009).

I thank my colleagues at TechnoPhage, S.A. for providing technical support, especially the stimulating team exercises aimed at developing solutions to the identified problems and for the sleepless nights we worked together. I am deeply indebted to my colleague and co-author, Clara Leandro, for her extremely valuable support and insights, without which this thesis would not have been possible. I would also like to thank Carlos São José and Madalena Pimentel for offering thorough and excellent feedback on the papers and on an earlier version of this thesis.

The epidemiological study was made possible only by the commitment of all professionals in various health facilities. I would therefore like to thank all those involved at Associação Protetora dos Diabéticos de Portugal and Centro Hospitalar de Lisboa Central EPE. I am especially indebted to José Neves, whose constant, positive influence and implicit encouragement sustained me throughout the work for this PhD.

The animal studies were also possible only through the total engagement of the Instituto de Medicina Molecular Animal Facility and the Faculdade de Medicina da Universidade de Lisboa Experimental Surgery staff, as well as the technical support provided by the Instituto de Medicina Molecular BioImaging Unit and Histology Service staff. I especially thank Dolores Bonaparte, whose experience in animal research was essential for the development of the project.

All the laboratory work for the epidemiological study and the biofilm studies was done at the Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa. This was made possible by the unwavering support of Professor Cristina Lobo Vilela, whose positive attitude and enthusiasm were and continue to be a true inspiration.

I would also like to thank Dr.ª Alexandrina Quintino and Professor António Sousa Guerreiro, Heads of the Internal Medicine Department of the Centro Hospitalar de Lisboa Central, and Rita Barata Moura, my clinical supervisor, for their constant encouragement and for giving me enough time to complete the thesis.

Finally, I want to thank my family. My beloved wife Natália and our sons João and António are a continuous and powerful source of inspiration and energy. I extend my special gratitude to my grandparents, parents, and sister for their never-ending support.

List of Abbreviations

AB – alamarBlue
Abi – abortive Infection system
BT – bacteriophage therapy
CFU – colony-forming units
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
CRTB – clinically relevant tissue burden
DFI – diabetic foot infection
DFU – diabetic foot ulcer
DG – dermal gap
DM – diabetes mellitus
DNA – deoxyribonucleic acid
EG – epithelial gap
ESBL – extended-spectrum β-lactamase
ds – double-stranded
EMA – European Medicine Agency
EPS – extracellular polymeric substance
FDA – Food and Drug Administration
GT – granulation tissue
HCP – health care provider
HDI – hair-density index
ICTV – International Committee for Taxonomy of Viruses
IM – input multiplicity
i.p. – intraperitoneal
i.v. – intravenous
MDR – multi-drug resistant
MRCN – methicillin-resistant coagulase-negative *Staphylococcus* spp. other than *Staphylococcus epidermidis*
MRSA – methicillin-resistant *Staphylococcus aureus*
MRSE – methicillin-resistant *Staphylococcus epidermidis*
P.H.A.G.E. – Phages for Human Applications Group Europe
PDR – pan-drug resistant
RM – Restriction-Modification
SDI – skin-damage index
ss – single-stranded
Sie – superinfection exclusion system
TAT – topical antimicrobial therapy
TSA – tryptone soy agar
$TcPO_2$ – transcutaneous oxygen pressure
US – United States
VRE – vancomycin-resistant enterococci

List of Figures

List of Tables

List of Publications

This doctoral thesis is based on the following articles, which, as first author, I published in peer-reviewed journals:

Paper 1:

Mendes JJ, Marques-Costa A, Vilela C, Neves J, Candeias N, Cavaco-Silva P, Melo-Cristino J.
Clinical and bacteriological survey of diabetic foot infections in Lisbon.
Diabetes Res Clin Pract. 2012 Jan;95(1):153-61. doi: 10.1016/j.diabres.2011.10.001.
Epub 2011 Oct 21.
PMID: 22019426 [PubMed – indexed for MEDLINE]

JJM conceived the study and developed the data collection tools. JJM, AMC, JN and NC collected the data. CV contributed to the conception and design of the study and conducted the microbiological analysis. PCS and JMC helped with the writing of the manuscript. JJM analyzed the overall data and prepared the manuscript.

Paper 2:

Mendes JJ, Leandro C, Mottola C, Barbosa R, Silva FA, Oliveira M, Vilela CL, Melo-Cristino J, Górski A, Pimentel M, São-José C, Cavaco-Silva P, Garcia M.
In vitro design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections.
J Med Microbiol. 2014 May 28. pii: jmm.0.071753-0. doi: 10.1099/jmm.0.071753-0.
[Epub ahead of print]
PMID: 24869663 [PubMed – as supplied by publisher]

JJM and CL conceived and designed the overall project. CSJ, MP, and MG planned the experiments on bacteriophage isolation and characterization. MO and CLV planned the experiments on established biofilms. CL, RB, and FAS conducted the bacteriophage isolation, amplification, purification, and DNA extraction. CL and RB additionally performed the bioinformatics analysis and assessed the bacteriophages' host range and activity against planktonic cells. CM conducted the experiments on established biofilms. CSJ, MO, CLV, PCS, JMC, and AG contributed reagents, materials, and analysis tools and helped with the writing of the manuscript. CSJ, MP, and MG analyzed the data related to the bacteriophages' morphological and genomic characterization. JJM analyzed the overall data and prepared the manuscript.

Paper 3:

Mendes JJ,Leandro C, Bonaparte D, Pinto A.
A rat model of diabetic wound Infection for the evaluation of topical antimicrobial therapies.
Comp Med. 2012 Feb;62(1):37-48.
PMID: 22330650 [PubMed - indexed for MEDLINE]

JJM conceived and designed the overall project. JJM, CL and DB conducted the animal studies. AP conducted the histologic analysis. JJM analyzed the overall data and prepared the manuscript.

Paper 4:

Mendes JJ,Leandro C, Corte-Real S, Barbosa R, Cavaco-Silva P, Melo-Cristino J, Górski A, Garcia M.
Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds.
Wound Repair Regen. 2013 Jul-Aug;21(4):595-603. doi: 10.1111/wrr.12056.
Epub 2013 Jun 11.
PMID: 23755910 [PubMed - indexed for MEDLINE]

JJM and CL conceived and designed the overall project. JJM, CL and RB conducted the experiments. PCS, JMC, and AG contributed reagents, materials, and analysis tools and helped with the writing of the manuscript. JJM analyzed the overall data and prepared the manuscript.

Parts of the introduction are also based on the following publication:

Mendes JJ, Neves J.
Diabetic foot infections: Current diagnosis and treatment.
The Journal of Diabetic Foot Complications. 2012;4(2): 26-45.

JJM prepared the manuscript. JN reviewed the manuscript.

Section A

Aims and Thesis Outline

Aims

The main objective is the development of a topically delivered bacteriophage treatment protocol (including formulation and dosage regimen) with potential efficacy in diabetic wound infections.

The secondary objectives are the following:

- conduct an epidemiological survey of diabetic foot infections in Lisbon, allowing the identification and characterization of the main bacteria involved and their correlation with clinical data;

- investigate, in vitro, the antimicrobial activity of bacteriophage solutions against planktonic cells and established biofilms;

- develop an animal model of diabetic wound infection suitable for the evaluation of topical antimicrobial therapies, namely topical bacteriophage therapy; and

- investigate, in vivo, the antimicrobial activity and wound-healing capability of bacteriophage solutions delivered topically to wounds in an animal model of diabetic chronic wound infection.

Thesis outline

This thesis is divided into three sections (A, B, and C).

Section A includes the aims and outline of the thesis as well as the general introduction, which describes the context of the research, providing a theoretical foundation for the thesis. The **General Introduction** presents background information on chronic wounds, specifically diabetic foot infections, and provides the current framework for their diagnosis and treatment. In addition, it gives a general outline of bacteriophage biology and bacteriophage therapy, especially concerning topical therapy.

Section B, whose general outline is presented in Figure A0-1, is divided into four chapters. **Chapter 1** presents the epidemiological survey of diabetic foot infections in Lisbon, allowing the identification, characterization and clinical correlation of the main bacteria involved, and thus justifying the chosen bacterial targets. **Chapter 2** presents a detailed characterization of the bacteriophages used with regard to their spectrum of activity, and their genetic and morphological structure. It also describes their activity against planktonic cells and established biofilms, justifying the posology and dosage regimen used in the animal studies. **Chapter 3** presents the optimization of a new wound infection model in chemically induced diabetic Wistar rats, which is suitable for the evaluation of topical antimicrobial therapies, namely bacteriophage therapy. **Chapter 4** presents the study of the antimicrobial and wound-healing capability of bacteriophage solutions delivered topically to wounds in two (rodent and porcine) animal models of diabetic chronic wound infection**.** Each chapter is introduced by a short text that frames it according to the general outline of the thesis.

Section C includes chapters on the general discussion and future research. The chapter on **General discussion** summarizes and discusses the main findings, as well as the implications and methodological limitations of the results obtained. The chapter on **Future prospects** presents future research directions that could provide the next steps along the path to a practical and widely applicable topical bacteriophage therapy. In addition, it also presents potential applications and future research directions outside the area of bacteriophage therapy.

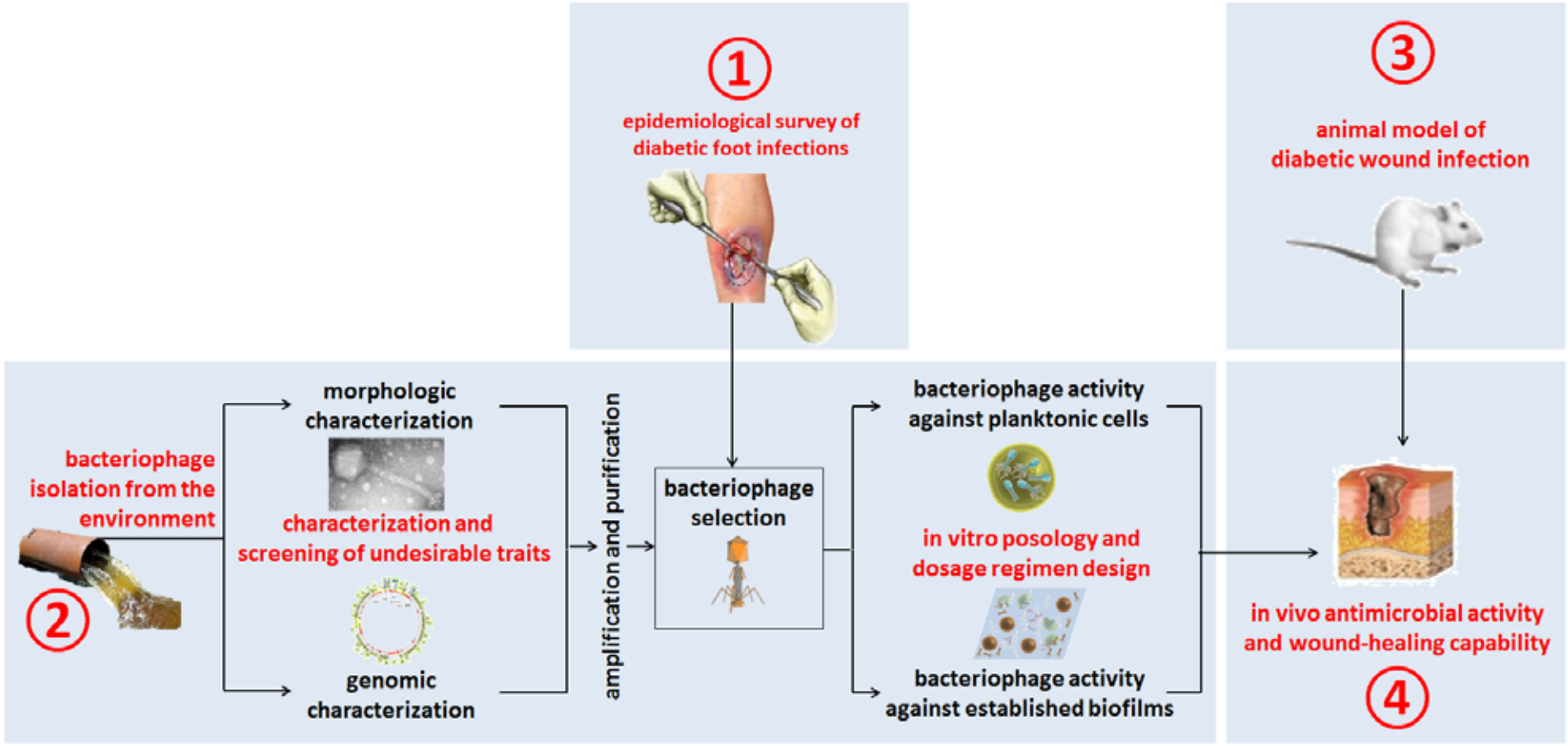


Figure A0-1 – General outline of Section B of the thesis. Each numbered shaded area corresponds to a chapter. For additional details please refer to the text.

General Introduction

Chronic wounds

Definitions and epidemiology

Chronic wounds, such as diabetic foot ulcers (DFUs), chronic venous leg ulcers, and pressure ulcers, are defined as wounds that have failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity over a period of 3 months (1). It has been estimated that around 1% of the population is affected by non-healing wounds, and the most affected population is over the age of 65 (2). Therefore, future aging trends in the world's population are likely to lead to a significant increase in the incidence of these wounds over the next 20 years. This is of particular relevance because chronic wounds are a major source of pain and disability in patients, and their treatment involves considerable economic cost (3).

Healing pathophysiology

Human skin is a complex and uniquely constructed organ with vital functions. Wounds occur when the integrity of the skin tissue is disrupted. In the acute wound setting, when this protective barrier is broken, the physiologic process of healing is immediately set in motion. In this classic model (4), wound healing is divided into three sequential but overlapping phases: (1) the inflammatory phase; (2) the proliferative phase (re-epithelialization, granulation and neo-angiogenesis); and (3) the remodeling phase (extracellular matrix remodeling). This complex, orchestrated biochemical cascade is characterized by signature events, cells, and their molecular regulators. For a complete review of the various cellular and inflammatory pathways involved in acute wound healing please refer to Schreml et al. (5).

Although most acute wounds heal in an uncomplicated fashion, following the aforementioned orderly and timely repair process, a large number of non-healing wounds fail to establish a sustained anatomic and functional result (6). Despite the differences in origin, non-healing wounds display common clinical features, include the following: the presence of necrotic tissue, lack of adequate blood supply, and excess exudate (7). Continuous bacterial clinical or sub-clinical infections (8) limit the cytokine-mediated switch to the later granulation tissue formation phase, resulting in prolonged inflammation and increased neutrophil infiltration with consequent protease activity. A persistent inflammatory phase is commonly witnessed by histopathology, which is associated with a delay in the formation of mature granulation tissue and failure of re-epithelialization (9).

Bacterial infection

As previously noted, clinical or subclinical infection is considered a common reason for impaired wound healing (10). Recent studies have emphasized both qualitative and quantitative aspects of wound microbiology, as well as the host's immune response, as critical determinants of wound outcome.

In relation to qualitative aspects, although important differences exist in the microbiology of various chronic wounds, some common concepts can be presented (Figure A1-1). Gram-positive cocci, namely *Staphylococcus aureus*, are the first microorganisms to colonize[1] and acutely infect[2] breaks in the skin. Chronic wounds develop a complex polymicrobial microbiology, including aerobic gram-negative rods and anaerobes. Nonfermenting gram-negative bacilli are found in many patients

[1] Presence of multiplying bacteria in a wound but not causing tissue damage
[2] Presence of multiplying bacteria in a wound causing tissue damage and clinical signs of infection

with chronic or previously treated infections, and *Pseudomonas aeruginosa* is specifically associated with wounds treated with wet dressings (11). Anaerobes are rarely the sole pathogen, but they often participate in a mixed infection with aerobes, especially in cases of deep tissue infection (12). These mixed infections provide an optimal opportunity for microbial synergy, which increases the net pathogenic effect and hence the severity of infection (13). Thus, the presence of specific pathogens is less important than are the composition of the polymicrobial wound microbiota and the presence of additional potentiating factors, namely bacterial biofilms. Bacterial biofilms are formed when planktonic phenotype bacteria attach to the wound surface and colonize into highly organized structures composed of extracellular polymeric substance (EPS) produced by the microorganisms and the host's surrounding tissues (14). Within the biofilm, both the protective outer coating and the altered bacterial phenotype contribute to the enhanced resistance of microorganisms to the host response, as well as to various antibiotic treatments. The described formation and behavior of the entire biofilm community is directed by signaling molecules that are produced when microorganisms reach a critical number—critical colonization[3]. This phenomenon is termed "quorum sensing," and it has been shown to be a key regulator of the expression of virulence factors as well as a modulator of host immunity (15).

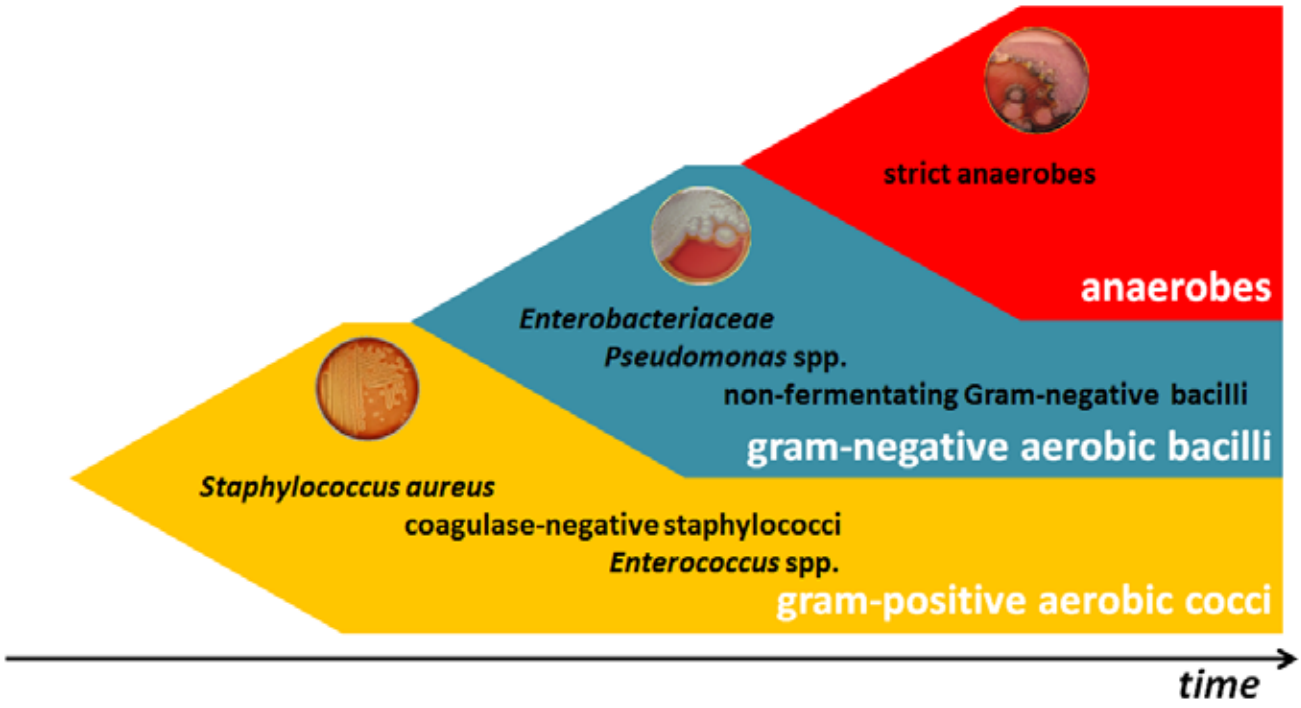


Figure A1-1 – Qualitative aspects of acute and chronic wounds. Gram-positive cocci, namely *S. aureus*, are the first microorganisms to colonize and acutely infect breaks in the skin. Chronic wounds develop a complex polymicrobial microbiota, including aerobic gram-negative rods and anaerobes.

Assuming that the qualitative microbiology remains constant, the probability of wound infection increases as the microbial load increases to a critical level, when infection or a failure to heal is considered almost inevitable (13). Breidenbach et al. (16) established this critical level in complex extremity wounds, as a bacterial tissue count >10^5 of colony-forming units (CFU)/g. However, there are exceptions to this rule of thumb because various organisms have different intrinsic virulence

[3] Presence of multiplying bacteria in a wound adversely affecting wound healing while not causing classical clinical sings of infection

potentials. A good example is *ß*-hemolytic streptococci, which are able to induce tissue damage at 10^2 CFU per gram of tissue, while greater counts of less pathogenic organisms may be of little clinical significance (17).

Another critical factor is the efficacy of the host's immune response in dealing with wound microbiota. Infection is facilitated by local potentiating factors, such as tissue necrosis and hypoxia (caused by poor local perfusion accentuated by the hypermetabolic state and microbial cellular metabolism), which impair the immune cell activity in the wound environment (18).

All these complex interactions have been systematized in the wound infection continuum (Figure A1-2). This concept describes the effects of increasing bacteria quantity and diversity in wound tissue and their relationship to the quality of the host's immune response (19).

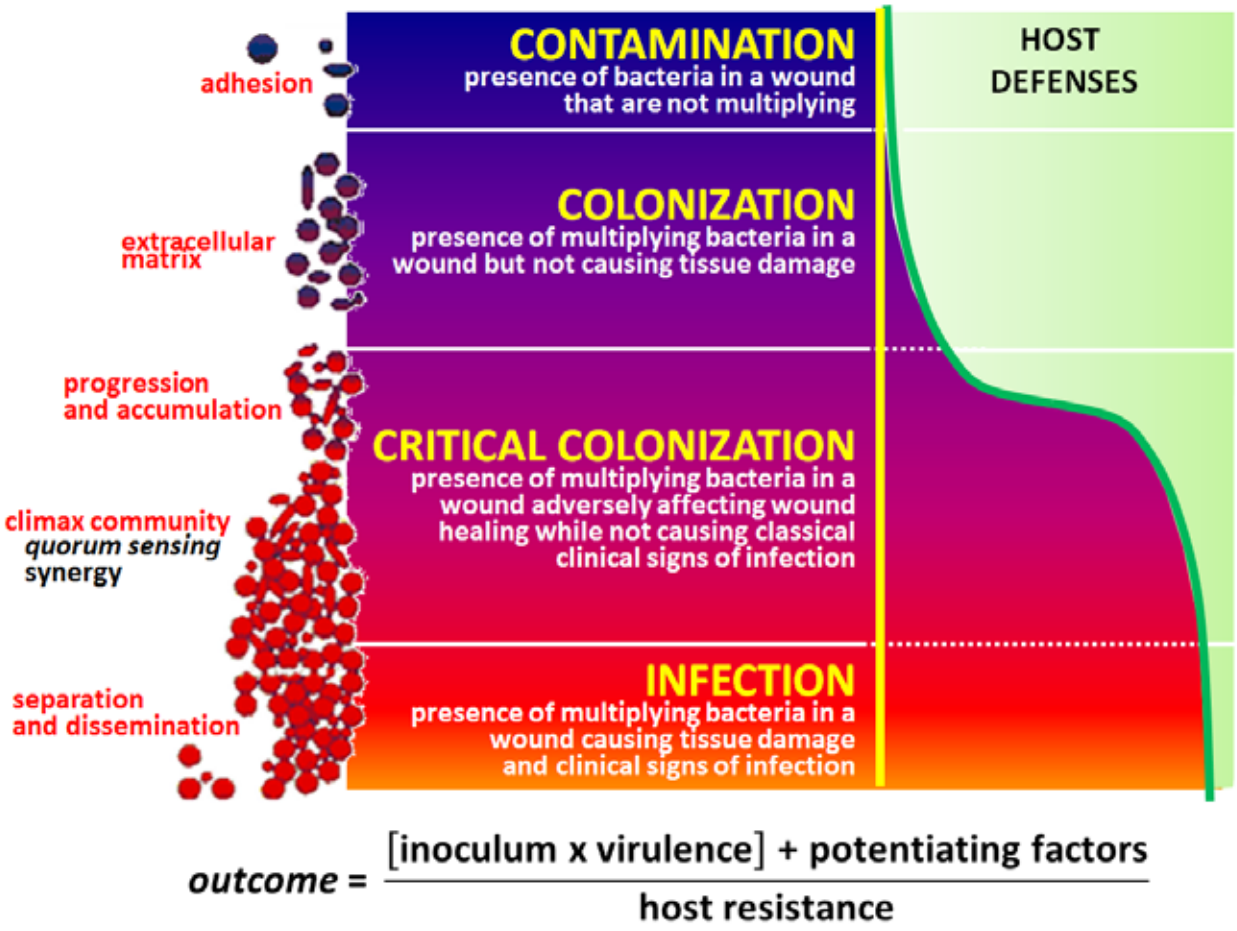


Figure A1-2 – Wound infection continuum. Wound infection can be defined as the process by which organisms bind to tissue, multiply, and then invade tissue and elicit an immune response. It can be illustrated as an infection continuum or shown as an equation. The quantity of microbes representing the states of colonization, critical colonization, and infection are unique and related to the quality of the host immune response. At a certain quantity, these organisms may begin quorum sensing or communicating chemically, triggering the expression of virulence factors, namely the production of biofilm.

Wound infection management principles

Correctly identifying the etiology of a chronic wound, as well as the local and systemic factors that may contribute to poor wound healing, is essential to an adequate therapy and key in successful

wound management (1). However, regardless of the specific type of wound, general wound management principles exist for all chronic wounds (20) (Figure A1-3).

The initial and most important step in the management of any chronic wound, infected or not, is to remove the necrotic material and debris until normal tissue appears. This is called debridement, which reveals the healthy tissue required for wound healing while the wound bed is cleansed of bacterial biofilms (21). Although there are several modalities of debridement, sharp debridement is generally regarded as fast and effective and, as elegantly demonstrated by Wolcott et al. (22), also opens a time-dependent antimicrobial therapeutic window. A serial debridement strategy further enhances this effect, by enabling the frequent disruption of the biofilm and increasing its vulnerability to treatment (23). This strategy is not only theoretical but also has been recognized as effective in a retrospective analysis of patients in a randomized controlled trial of growth factor therapy in DFUs (24).

Antimicrobial therapy may further enhance the reduction of the number of bacteria in chronic wounds (25). Although systemic infection is treated with systemic antibiotics (13), these do not effectively decrease bacterial levels in granulating wounds where, theoretically, topically applied antimicrobials (topical antibiotics or antiseptics) could be more effective (26). Irrespective of the definitions of antibiotics and antiseptics, for which a lack of consensus within the literature exists, these may be divided into two categories: antimicrobial solutions used to irrigate wounds and antimicrobial preparations designed for longer periods of contact time. The former usually have only a brief contact time with the wound surface and include hypochlorites and substances, such as potassium permanganate. The latter are normally developed as creams, ointments, and impregnated dressings, including most topical antibiotics (e.g. fusicidic acid) and silver-based products (e.g. silver sulphadiazine). Some products (available in different forms) fall into both categories: povidone iodine, chlorhexidine and hydrogen peroxide (27). Controversy has long surrounded the use of topical antimicrobial agents because of the lack of adequate proof of their efficacy, reports of cytotoxicity, and risk of antibiotic-resistance induction, which depends on the particular formulation, concentration of active ingredient, and duration of exposure (28). Most efficacy studies are suboptimal and have varied designs that are not easily comparable (28). Moreover, most have not considered or have even excluded debridement (27). However, a systematic review of controlled trials (29) showed that several topical substances hastened healing and induced a few improved outcomes. A recent Cochrane systematic review (30) concluded that evidence supported the use of a topical antiseptic in a specific chronic wound type. Regardless of the decision to initiate topical antimicrobial therapy, general consensus exists about its discontinuation when bacterial balance has been achieved because protracted courses of antibiotics may inhibit wound healing and promote the development of resistant organisms (20).

After preparation of the wound bed by using debridement and antimicrobial agents, a moist environment, which has been accepted as the best topical environment for open wounds (20), should be maintained. If, despite all efforts of optimization, the wound fails to heal in a timely fashion, surgical closure is generally recommended.

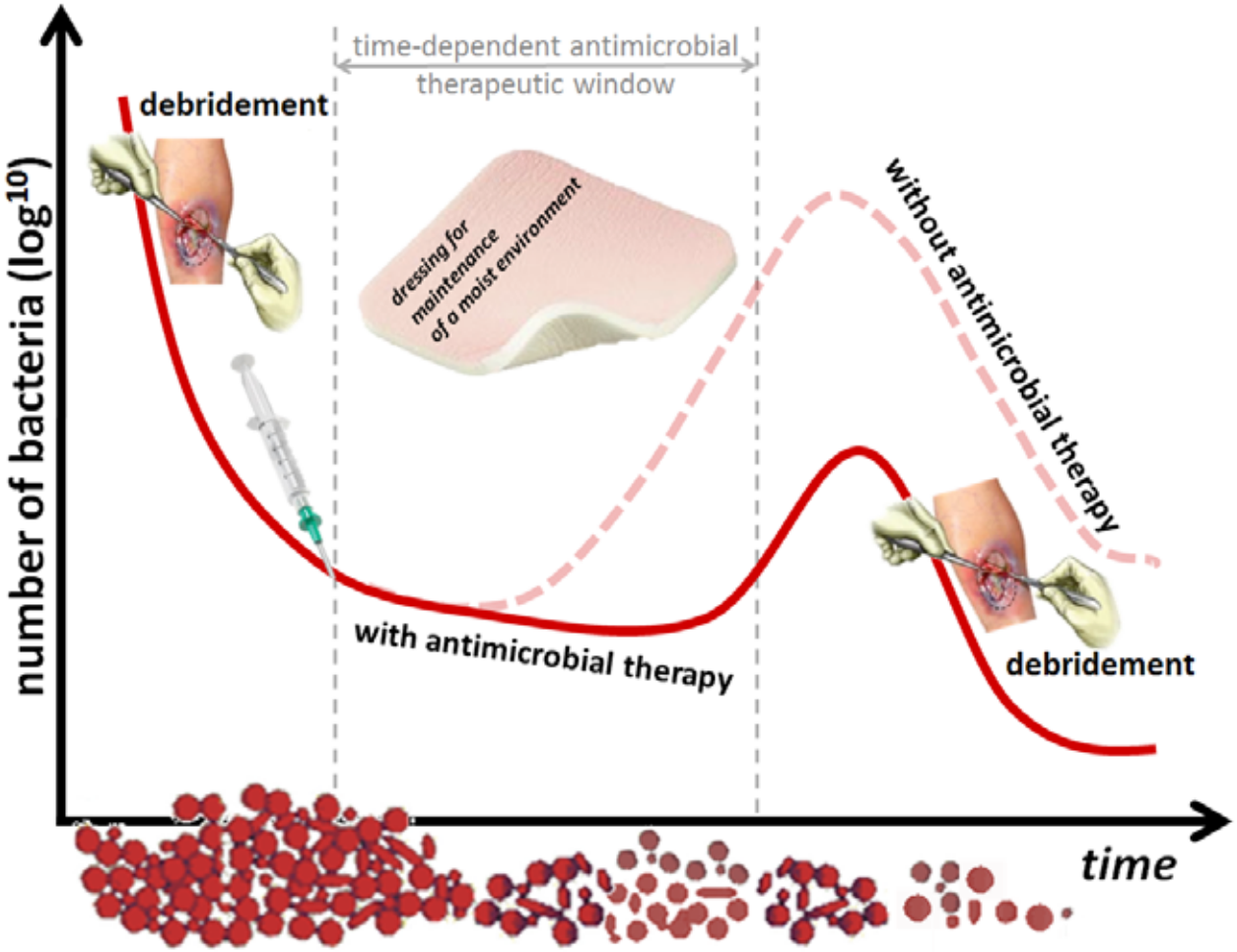


Figure A1-3 – Common wound infection management principles. The initial and most important step in the management of any chronic wound is debridement, which opens a time-dependent antimicrobial therapeutic window. Antimicrobial therapy (topical antibiotics or antiseptics) may further enhance the reduction of the number of bacteria in chronic wounds.

Diabetic foot infections

Definitions and epidemiology

The world is facing a major epidemic of diabetes mellitus (DM). There are an estimated 171 million diabetic patients worldwide, and this number is expected to double by the year 2030 (31). All DM patients are at risk of developing a diabetic foot ulcer (DFU), which is a full-thickness wound below the ankle, irrespective of duration. Based on current studies, the annual population-based incidence is 1 to 4% with a prevalence of 4 to 10%, and the estimated lifetime risk is 25% (32). According to a study published by the Eurodiale study group (33), approximately 58% of DFU patients will become clinically infected. Patients with DM frequently require minor or major amputations of the lower limbs (15 to 27%), and in more than 50% of cases, infection is the preponderant factor (34). Major amputation is associated with significant morbidity and mortality (ranging from 13 to 40% at 1 year to 39 to 80% at 5 years (32)), in addition to immense social, psychological and financial consequences (35). The treatment of diabetic foot infections (DFI) accounts for up to one-quarter of all diabetic admissions in both Europe and the United States (US), making it the single most common reason for DM-related hospital admission (36). The solution to this predictable progression includes the development of structured screening tools to identify those at risk and the implementation of standardized education and prevention protocols. However, as stated by Lavery et al. (37), even with the best preventive standard of care, 9% of patients with DM will still develop a DFI, with the consequent risk of amputation.

Pathophysiology

A prior DFU is an almost obligatory prerequisite for DFIs, even though, in some cases, the wound may have closed before DFI presentation (37). DFUs have a multifactorial nature, as numerous observational studies have indicated. It is well established that insulin deficiency (absolute or relative) is the basis of the biochemical abnormalities that lead to the organic complications of DM and the biological deficits of tissue healing and regeneration (38). DFUs result from a complex interaction of two major risk factors: neuropathy—both symmetric and bilateral, with varying degrees of alterations in autonomic, sensory and motor functions —plays the main role, while peripheral vascular disease resulting from atherosclerosis plays a secondary role (Figure A1-4). Approximately 50 to 60% of all DFUs can be classified as neuropathic. Signs or symptoms of vascular compromise are observed in 40 to 50% of all patients. The vast majority of patients have neuroischemic ulcers, and only a minority of patients have purely ischemic ulcers (39). However ulceration of the diabetic foot, either neuropathic or ischemic, does not occur spontaneously; it follows some form of extrinsic (e.g., low-pressure trauma from ill-fitting shoes) or intrinsic trauma (e.g., from the atrophy induced by motor neuropathy of the foot's intrinsic muscles) (40).

Once the protective layer of skin is broken, deep tissues are exposed to bacterial colonization. In DFUs, in addition to the considerations discussed in the previous chapter, infection is facilitated by ischemia, the particular anatomy of the foot (i.e., it is divided into several compartments, which explains the rapid spread of infection), and intrinsic immunological deficits, especially in terms of neutrophil dysfunction (41).

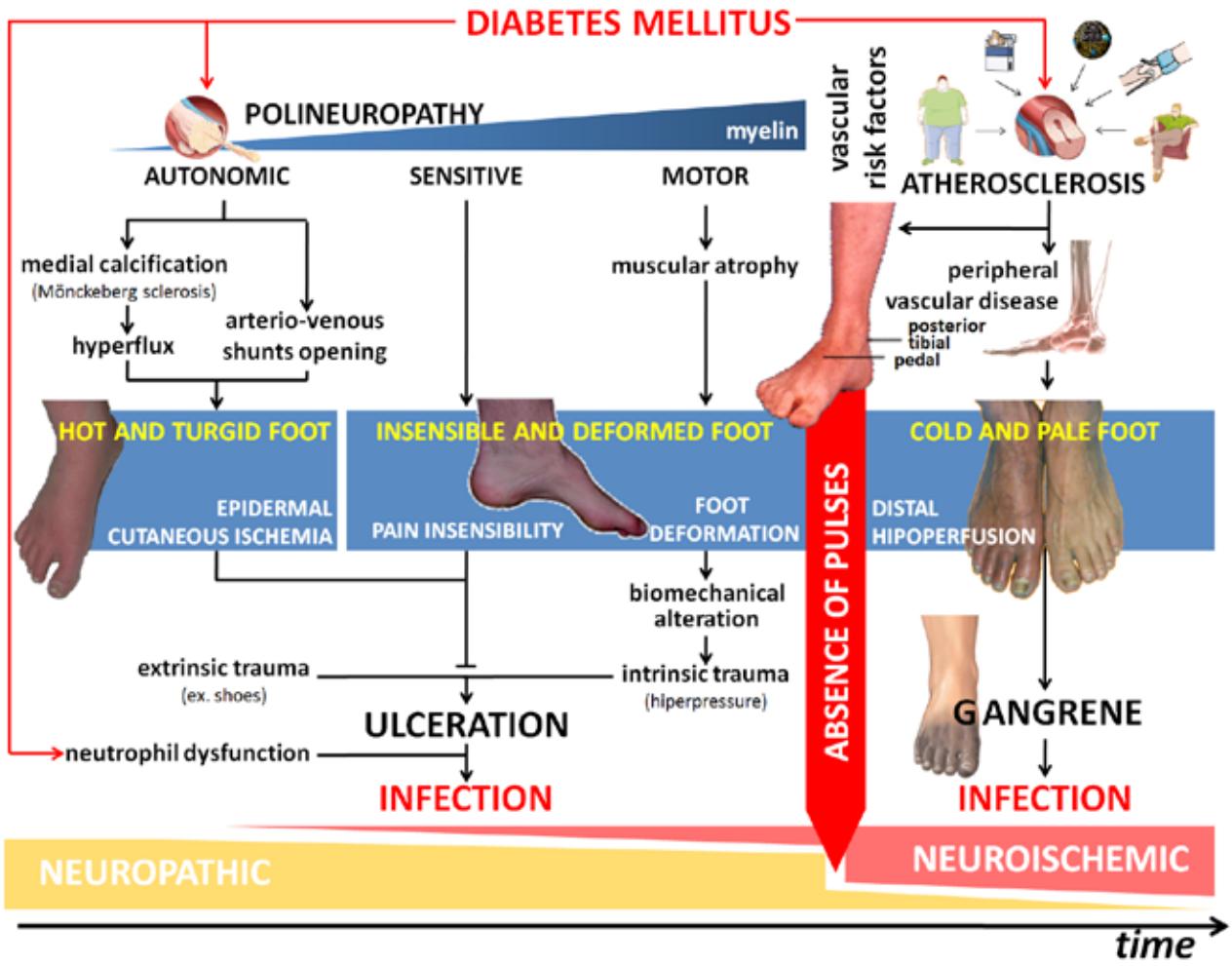


Figure A1-4 – Diabetic foot infection pathophysiology. Diabetic foot ulcer results from the complex interaction of a number of risk factors. Neuropathy (with alterations in motor, sensation, and autonomic functions) plays the central role and causes ulcerations because of trauma or excessive pressure in a deformed foot without protective sensibility. Once the protective layer of skin is broken, deep tissues are exposed to bacterial colonization. Infection is facilitated by diabetes mellitus-related immunological deficits, especially in terms of neutrophils, and it rapidly progresses to the deep tissues.

Bacterial infection

The microbiologic features of DFIs develop according to the already described microbiological principles of chronic wounds, with some specificities. Acute infections in patients who have not recently received antimicrobials are often monomicrobial (almost always with an aerobic gram-positive cocci), whereas chronic infections are often polymicrobial (including gram-positive and gram-negative aerobes and anaerobes (18)). However, the impaired host's defenses around necrotic tissue allow low-virulence bacteria, such as coagulase-negative staphylococci and *Corynebacterium* species, to assume a pathogenic role (11).

The intrinsic pathophysiological characteristics of DFIs, along with long periods of hospitalization, complex surgical procedures, and prolonged broad-spectrum antibiotic therapy, are also predisposed to infection with antibiotic-resistant organisms (e.g., methicillin-resistant *S. aureus* [MRSA] or vancomycin-resistant enterococci [VRE]) (18, 42).

Assessment

Recognizing important risk factors and making a logical treatment-oriented assessment of DFIs requires a consistent and thorough diagnostic approach. Such evaluation involves the careful assimilation of global medical foot and wound history, a systemized and detailed physical examination, and the results of complementary diagnostic procedures. Various systems have been proposed to classify DFUs, but none has gained widespread acceptance. The International Working Group of the Diabetic Foot developed the PEDIS classification system (43), which consists of internationally applicable guidelines that can reliably predict the outcome of diabetic foot management (44). The PEDIS system classifies all DFUs into the subcategories of five main parameters (perfusion, extent/size, depth/tissue loss, infection and sensation), according to strict criteria (Table A1-1). Although it was not developed as a guide for daily management or to predict the outcome of an individual patient, it considers all the potentially useful information obtained from the patient's clinical history, foot examination, and diagnostic exams. Consequently, the use of this systematic examination ensures that important aspects are not overlooked.

The assessment of DFIs based on the PEDIS classification has been reviewed elsewhere (45). In this chapter, we will consider only the evaluation of infection. The diagnosis of infection is clinical, based on the presence of symptoms and signs of inflammation (39), and it must always be confirmed and classified according to the depth of involvement. In the PEDIS grading system, three parameters are particularly relevant to clinical management and outcome: the involvement of skin only (Grade 2); the involvement of deeper structures (Grade 3); and the patient's systemic inflammatory response (Grade 4). To categorize the patient definitively into one of these groups, different diagnostic procedures are indicated.

All patients should have a complete blood count with differential, erythrocyte sedimentation rate and c-reactive protein testing. However, caution must be exercised when interpreting laboratory tests because no marker is sufficiently sensitive or specific to confirm the diagnosis of DFI, and tests are often misleading, even in the case of severe lesions (46). In these patients, the most sensible sign of infection is often recalcitrant hyperglycemia, despite regular anti-hyperglycemic regimens.

Another problem is determining the presence of osteomyelitis. The International Working Group on the Diabetic Foot has proposed consensus criteria (47) for diagnosing diabetic foot osteomyelitis; however, these remain to be validated by a properly designed trial. A positive probe-to-bone test (i.e., when a sterile metal probe reveals a hard and gritty surface compatible with bone) in the presence of DFI appears to have a relatively variable positive predictive value, while a negative test in a low-risk patient markedly decreases the likelihood of osteomyelitis (48). This simple technique may be complemented by imaging studies (e.g., plain radiographs and magnetic resonance imaging). However, the gold standard criterion for diagnosing osteomyelitis is a characteristic histopathology (acute or chronic inflammatory cells or necrosis) associated with a positive culture from a bone specimen ideally obtained at the time of surgical debridement or by fluoroscopic- or computed tomography-guided percutaneous biopsy (47).

Table A1-1 – PEDIS classification system. The International Working Group of the Diabetic Foot's PEDIS system classifies all foot ulcers in subcategories of five main categories (perfusion, extent/ size, depth/tissue loss, infection and sensation), according to strict criteria. $PaCO_2$: partial pressure of carbon dioxide in the arterial blood; TcpO2: transcutaneous oxygen pressure

Categories	Grades	Description
Perfusion	**grade 1**	No symptoms or signs of peripheral artery disease in the affected foot in combination with – palpable dorsal pedal and posterior tibial artery *or* – ankle-brachial index 0.9 to 1.10 *or* – toe-brachial index >0.6 *or* – $TcpO_2$ >60 mmHg
	grade 2	Symptoms or signs of peripheral artery disease but not of critical limb ischemia: – presence of intermittent claudication *or* – ankle-brachial index < 0.9, but with ankle pressure >50 mmHg *or* – toe-brachial index<0.6, but systolic toe blood pressure >30 mmHg *or* – $TcpO_2$ 30-60 mmHg *or* – other abnormalities on non-invasive testing, compatible with peripheral artery disease but with critical limb ischemia
	grade 3	Critical limb ischemia: – systolic ankle blood pressure <50 mmHg *or* – systolic toe blood pressure <30 mmHg *or* – $TcpO_2$ < 30 mmHg
Extent	Wound size after debridement (measured in square centimeters)	
Depth	**grade 1**	Superficial full-thickness ulcer, not penetrating deeper than the dermis
	grade 2	Deep ulcer, penetrating to subcutaneous structures (fascia, muscle or tendon)
	grade 3	Deep ulcer, penetrating any of the subsequent layers of the foot (bone or joint)
Infection	**grade 1**	No symptoms or signs of infection
	grade 2	Infection involving the skin and the subcutaneous tissue only at least two of the following items must be present: – local swelling or induration – erythema >0.5 to 2 cm around the ulcer – local tenderness or pain local warmth – purulent discharge (thick, opaque to white or sanguineous secretion)
	grade 3	Infection involving structures deeper than skin and subcutaneous tissues *or* erythema >2 cm plus one of the items described above
	grade 4	Foot infection accompanied by signs of systemic inflammatory response syndrome: – temperature >38 ºC or <36 ºC – heart rate >90 beats/min – respiratory rate >20 breaths/min (or $PaCO_2$ <32 mmHg) – white blood cell count >12.000 or <4.000 cells/mm^3 (or 10% band forms)
Sensation	**grade 1**	No loss of protective sensation
	grade 2	Loss of protective sensation absence of perception of the one of the following tests: – absent pressure sensation, determined with a 10-g monofilament on two out of three sites on the plantar side of the foot *or* – absent vibration sensation, determined with a 128-Hz tuning fork tested on the hallux

In the absence of suspected osteomyelitis, bacteriological sampling, which must be done after mechanical debridement and cleansing of the wound with gauze soaked in sterile physiological saline, is indicated if infection is clinically suspected. The best sampling technique remains a matter of debate. While tissue biopsy and fluid aspirate are considered the gold standard for diagnosing wound infection (13), such invasive tests are infrequently performed for superficial wounds or in many practice settings, such as outpatient clinics, because of concerns about enlarging the ulcer or inducing pain (13, 49). Superficial swabbing of the wound is discouraged, but swabbing the base of the ulcer is acceptable if it is the only possible option (50). Independent of the sampling method, specimens must be placed in transport medium and be sent to the microbiology laboratory as quickly as possible. Assuming that at present, there are no completely reliable microbiological methods to distinguish between pathogenic and nonpathogenic microorganisms, microbiologists and clinicians must collaborate closely to interpret the results, taking into account the sampling conditions, transport time and conditions, and the type of bacteria isolated.

Treatment

When a DFI patient presents to the care team, a multidisciplinary management strategy should be rapidly implemented (Figure A1-5) because evidence suggests that this reduces the incidence of major amputation. The multidisciplinary team should include the following: a diabetologist, a surgeon with relevant expertise in managing DFI, a tissue viability nurse and ideally a podiatrist, as well as access to other specialist services (e.g., vascular surgeons and orthopedists) (50).

The literature includes excellent, complete reviews on the current treatment strategies of DFIs (18, 45), which is beyond the scope of this thesis. In this chapter, we will consider only infection control. Drainage and surgical debridement are two different but complementary surgical procedures that are essential in infection control. Drainage is the incision of an area of tissue phlegmon or abscess, and should be the first-line treatment for all DFIs, if these are present. Debridement, following the principles already described, should follow and be performed as soon as possible.

Randomized clinical trials have shown that systemic antibiotics selected according to the severity of infection are clinically valuable in DFIs (18, 51); as in the majority of infectious diseases, they must be provided as early as possible. However, as authoritative guidelines emphasize (18, 50) and a recent systematic review confirms (52), no particular antimicrobial regimen has been shown to be superior to others in DFI treatment. The initial empirical antibiotic therapy in DFIs should aim to cover the most common pathogens, based on the known local epidemiology of DFIs. Moreover, the therapy and should subsequently be refined according to clinical response and microbiological results (11). The optimal duration of antibiotic therapy has not been clearly established, but it could be 1 to 2 weeks for simple forms of infection, and 2 to 4 weeks for moderate to severe forms of skin and soft tissue infections (18). The application of topical antibiotics, although not currently advisable for most clinically infected chronic wounds, may be considered for a properly managed wound with subclinical infection that is failing to heal, or to help in the removal of biofilms that have been implicated in persistent infections (28).

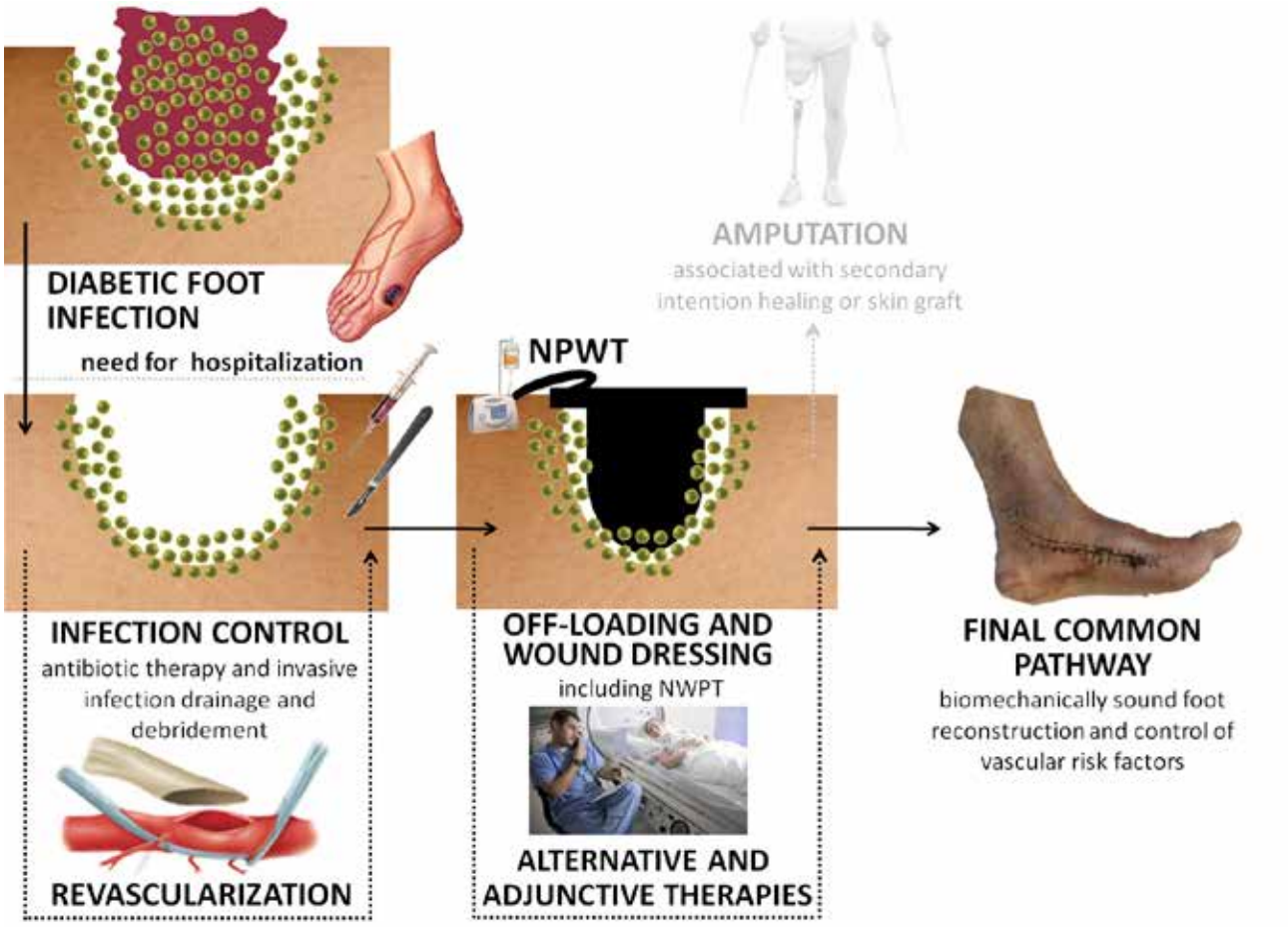


Figure A1-5 – Management of diabetic foot infections. The multidisciplinary team must consider draining invasive infections, debriding necrosis, and promptly starting empirical antibiotic therapy, complemented by appropriate vascular reconstruction. Complete and permanent off-loading of the wound should follow. Accumulating evidence indicates that negative pressure wound therapy (NPWT) should be included in the treatment pathway. Assuming appropriate attention to all these steps, a wound that fails to improve should prompt the clinician to consider alternative and adjunctive therapies. Control of vascular risk factors and a biomechanically sound surgical reconstruction, with or without amputation, must be considered in the final common pathway of the treatment plan in order to minimize the risk of recurrent ulceration.

Once the infection has been controlled, revascularization must be immediately considered. The main objective in patients with DFIs is to obtain sufficient perfusion to control the infection and save the limb, in which the temporary improvement of perfusion obtained with endovascular therapy may be sufficient to promote healing and prevent amputation (53).

After the wound has healed, the control of vascular risk factors should be addressed. In the presence of deformity, a biomechanically sound foot reconstruction must be completed to prevent the recurrence of foot ulceration. On the other hand, if the wound fails to improve despite repeated surgical interventions, alternative and/or innovative therapies should be considered (e.g., growth factors and hyperbaric oxygen therapy). However, if all these treatments fail or are not considered, amputation remains the only option in cases of severe infection, especially in the neuroischemic foot. Major (leg or thigh) amputations should be exceptional, occurring only in cases of uncontrolled life-threatening infection.

Bacteriophages

Introduction

Bacteriophages are viruses that consist of a genome contained within a protein coat and specifically infect bacteria. They are the most abundant entities on earth (the estimates range from 10^{30} to 10^{32} (54)), and they play key roles in regulating the microbial balance in every ecosystem where this has been explored (55).

Bacteriophages were discovered independently by two microbiologists: in 1915 by the British Felix Twort (56) and in 1917 by the French-Canadian Felix d'Hérelle (57). Although Twort did not pursue his discovery, d'Hérelle systematically investigated the nature of bacteriophages (58) and explored their ability to function as therapeutic agents (59).

Bacteriophage taxonomy

As viruses that infect bacteria, bacteriophages are genotypic and phenotypically different from viruses that infect archaea (archaeovirus) and eukarya (eukaryovirus). The name "bacteriovirus" was recently proposed as scientifically appropriate (60). The classification of bacteriophage was assigned to the International Committee for Taxonomy of Viruses (ICTV), which recognizes 14 families of bacteriophages (61). Eleven of these families are not grouped in a superior taxonomical category, while the other three are included in the order *Caudovirales*. This comprises the vast majority of known bacteriophages (96 %) and its members have in common deoxyribonucleic acid (DNA) genomes and a complex morphology with a capsid of regular symmetry (the head) and a DNA injection apparatus of helicoidal symmetry (the tail). The morphology of the tail defines the three families of the order: *Myoviridae* (with a long, contractile tail), *Siphoviridae* (with a long, non-contractile tail), and *Podoviridae* (with a short tail).

The ICTV classification is based mainly on morphological analysis, nucleic acid type, and host organism. This classification has been greatly criticized during the past few years because it is dependent on electron microscopic images and does not take into account the rapidly increasing amount of genomic and proteomic data (62). Furthermore, innumerable bacteriophages whose genomes have been completely sequenced—especially prophages of lysogenic bacteria and bacteriophages of non-cultivable hosts—for which no electron microscopic images are available. Because bacteriophage genomes are highly mosaic, it is now becoming clear that a strictly hierarchical taxonomy cannot represent the complex relationships between viral species. Thus, there is increasing consensus that bacteriophage classification should be based on genomic data (63).

Bacteriophage lifecycle

Lysis of the host cell by bacteriophages is a complex process consisting of a cascade of events involving several structural and regulatory genes. Moreover, not all bacteriophages replicate in a similar way, and there are significant differences in their replication cycles between strictly lytic[4] and temperate bacteriophages. However, for a specific group of bacteriophages, *Caudovirales*, morphogenesis is so similar that a standard process has been suggested (64). Bacteriophages do not have their own metabolism, and require the metabolism, energy resources, and materials of their hosts to replicate. Common steps in the replication process of bacteriophages can be properly correlated with the graphical representation of the one-step growth curve, which translates the experimental interaction between the bacteriophage and its host over time (Figure A1-6).

[4] Strictly lytic (often described as virulent) bacteriophages are both lytic and incapable of displaying a lysogenic cycle. Throughout the text the term "lytic bacteriophage" refers only to "strictly lytic bacteriophage".

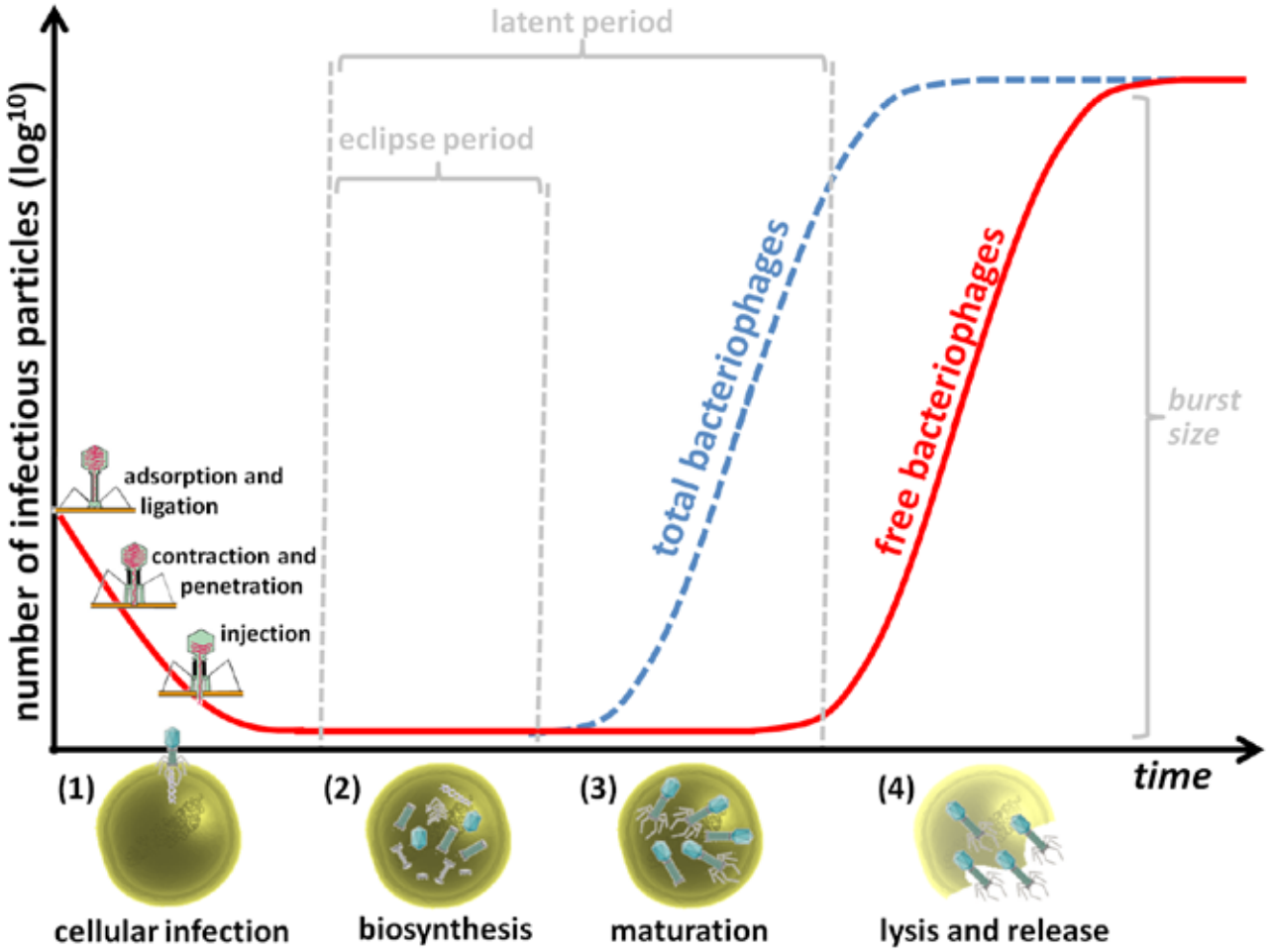


Figure A1-6 – Common steps of the bacteriophage lytic replication process properly correlated with graphical representation of the one-step-growth curve (a myovirus is used here as example). Once a bacteriophage encounters a target bacterium the process of bacteriophage replication takes place: (1) the bacteriophage adsorbs and ligates to bacterial surface receptors, then the sheath contracts and the hollow tail tube is thought to penetrate through the cell cytoplasmic membrane, injecting bacteriophage nucleic acid in the bacterial cytoplasm; (2) the genetic material of the bacteriophage takes up the biosynthetic machinery of the host and, during the eclipse period, mRNA expression occurs resulting in directed macromolecular biosynthesis; (3) during maturation, the previously synthesized bacteriophage structural proteins are assembled, and bacteriophage particles accumulate inside the cell; (4) at the end of the latent period, the accumulation of lytic proteins results in cell lysis and release of bacteriophages. The burst size corresponds to the average number of progeny bacteriophage particles produced per infected bacterium.

Cellular infection

Bacteriophages must encounter target bacteria before cellular infection occurs. This process of extracellular search occurs through diffusion and other means of movement (65, 66), which is followed by virion adsorption to the host bacteria. Adsorption is the process by which specific bacteriophage receptor binding proteins (tail fibers in *Myoviridae*), through the effects of diffusion and Brownian motion[5], come into contact with specific and chemically complementary locations (bacterial receptors) on the bacterial surface. Generally, after an initial weak and reversible interaction,

[5] Random movement of microscopic particles suspended in a fluid resulting from the impact of molecules of the surrounding medium

a strong and irreversible binding occurs between one or more components of the bacteriophage tail and a receptor of the cell surface (67). For this purpose, bacteriophages can use as specific receptors bacterial capsules, different parts of lipopolysaccharide, flagella, fimbriae, and many other surface proteins. The second step involves major structural rearrangements of the bacteriophage tail and tail/heath connector (tail contraction in the case of myoviruses), which culminate in the penetration (mechanic and/or enzymatic) of the tail apparatus through the host cell envelope (68). The third step is the injection of bacteriophage DNA into the bacterial cytoplasm.

Lytic cycle

After the injection of viral DNA, the genetic material of the bacteriophage takes up the biosynthetic machinery of the host (69). In the first phase, called the eclipse period, it is not possible to find bacteriophages inside or outside the cells. During this period, specifically ordered mRNA expression occurs, which results in directed macromolecular biosynthesis: (1) early protein synthesis, which constitutes a set of proteins required for replication of the bacteriophage genetic material; and (2) late proteins synthesis, corresponding to a set of structural proteins (comprising the capsid and the various components of the tail) and proteins required for the lysis step. The next phase is called maturation. During this period, the genetic material and the previously synthesized bacteriophage structural proteins are assembled, and bacteriophage particles accumulate inside the cell. The last phase, which corresponds to the end of the latent period, occurs because of the accumulation of lytic proteins and results in cell lysis and the release of bacteriophages (67, 69).

Temperate bacteriophages and lysogenic cycle

Temperate bacteriophages can multiply through the lytic cycle, or they may remain in a quiescent state inside the host cell. In this state, the bacteriophage genome (referred to as prophage) exists in a suppressed state, and the majority of genes are not transcribed. In many cases, the bacteriophage genetic material integrates into the host chromosome and is replicated and passed to daughter cells (67).

Bacteriophage-biofilm interactions

Despite the increasing recognition of the importance of bacteriophage-biofilm interactions, the complex nature of those interactions is not yet conclusively understood (70). Biofilms are dynamic structures containing voids and passageways lined with bacteria, with ongoing balances between bacterial growth, microcolony differentiation, and bacterial release into the planktonic state (71). Biofilms pose barriers to extracellular search because of its intricate structure, and the movement of bacteriophages from the bulk fluid to the biofilm is dependent on particle concentration (72). Bacteriophages can further increase their mobility within the biofilm by deploying EPS-degrading depolymerases, which can modify the biofilm's structure (73). The subsequent steps depend on the bacteriophage's encounter with susceptible bacteria in microcolonies. This is followed by host infection cycles and progeny release with continuous additional EPS depolymerization (Figure A1-7).

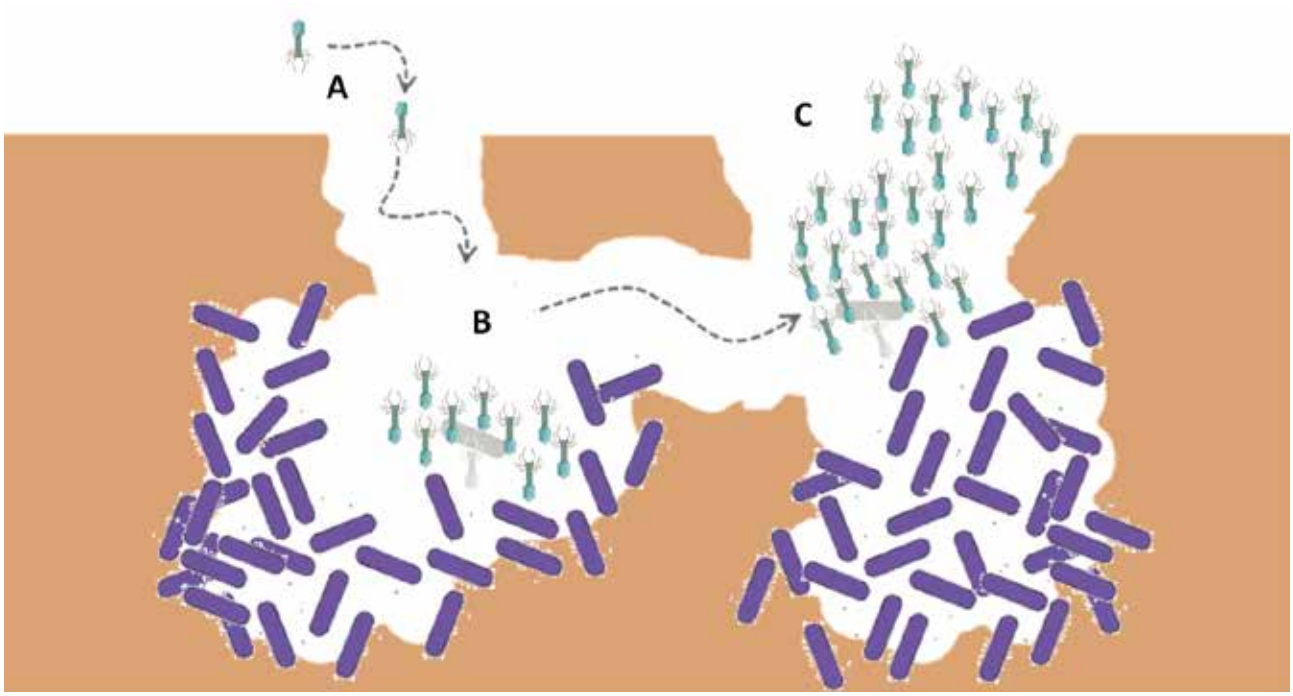


Figure A1-7 – Bacteriophage-biofilm interactions (adapted from (70)). Considered is a lytic bacteriophage that is physically associated with an extracellular polymeric substance (EPS)-depolymerase that interacts with an EPS-displaying biofilm: **(A)** a free bacteriophage diffuses and encounters a biofilm EPS, resulting in local digestion; **(B)** bacteriophage encounters and infects a susceptible bacterium in a microcolony, with subsequent progeny release; and **(C)** additional EPS depolymerization occurs along the path of a newly released single bacteriophage, which then encounters and infects a new microcolony associated bacterium, resulting in a progeny release that is followed by free-bacteriophage dissemination into bulk water toward subsequent biofilm acquisition.

Bacterial resistance to bacteriophages

Genotypic resistance

Bacteria are constantly challenged by bacteriophages and rates of bacterial bacteriophage resistance can be as high as 10^{-3} per host-cell, but usually average 10^{-7} (74). This resistance to bacteriophage infection is accomplished by mutation and selection, and it depends on a variety of mechanisms. These include the following: (1) blocking bacteriophage adsorption; (2) inhibiting the injection of bacteriophage genomes; (3) preventing DNA integration by superinfection exclusion systems (Sie); (4) degradation of bacteriophage DNA by the Restriction-Modification (RM) defense system and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR); and (5) blocking bacteriophage replication, transcription, translation or virions assembly by the abortive-infection system (Abi) (74). However, the most common mechanism resistant to bacteriophage infection involves strategies to prevent a bacteriophage from attaching to the cell surface, such as the lack of receptor expression, an alteration of a receptor, and the masking or shielding of receptors (75).

Evolutionary dynamics of bacteria and bacteriophage

It has been shown that bacteria and their corresponding lytic bacteriophages coevolve and establish equilibrium between their respective populations, in interactions that are analogous to those of a predator-prey model (76). Therefore, it is anticipated that natural bacteria-bacteriophage

populations, especially when in biofilm, will exhibit the coexistence of resistant and sensitive bacterial clones (76). This can be caused solely by a mutation and selection process, resulting in a constant arms race between bacteriophage and its host (77). However, there is evidence for non-heritable mechanisms that lead to the coexistence of bacteria and bacteriophages. Four equally valid and non-mutually excluding theories have been presented (Figure A1-8): numerical refuge, physiological refuge hypothesis, spatial refuge, and shielding by bacterial debris. The numerical refuge theory (78) predicts that simple mass-action interactions between bacteriophages and sensitive and resistant bacteria determine the stability of the population, so when densities of sensitive bacteria are low, bacteriophage densities will also decline, allowing sensitive cells to increase again. The physiological refuge hypothesis (79) postulates that during certain stages of bacterial life cycles sensitive bacteria may become transitorily resistant to bacteriophage infection, which may occur for example if resistance is based not on a complete absence of receptors, but instead on a greatly reduced number of receptors. The spatial refuge theory (80) states that physical heterogeneity in the environment protects some sensitive bacteria from bacteriophage infection. Finally, the shielding by bacterial debris predicts that active bacteriophages adsorb into fragments of lysed cells (debris) and inject their genetic material in a suicidal manner discounting from the system as a bacteriophage (81).

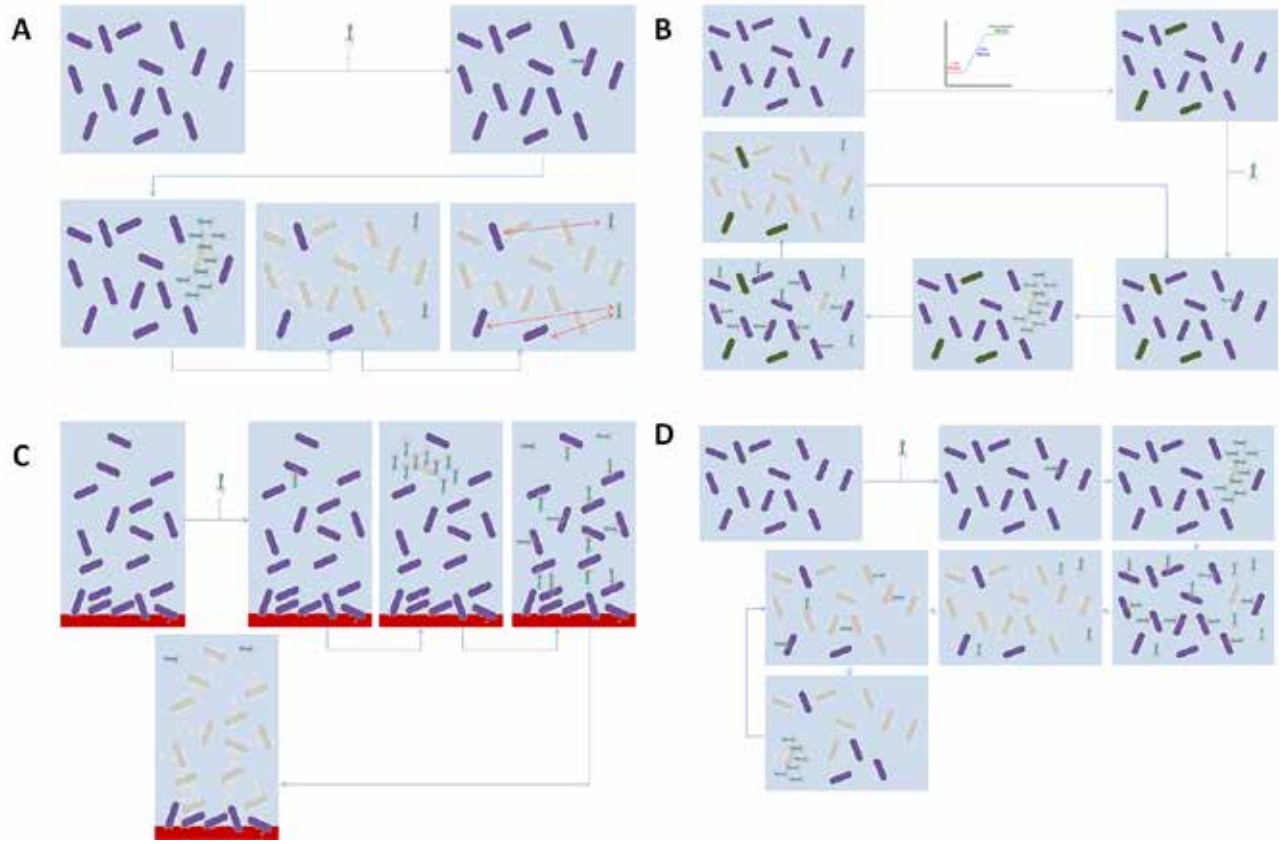


Figure A1-8 – Theories suggested to explain bacteria-bacteriophage coexistence and coevolution: **(A) numerical refuges** predicts that simple mass-action interactions between bacteriophages and sensitive and resistant bacteria determine the stability of bacteria and bacteriophage population; **(B) physiological refuges** postulates that during certain stages of bacterial life cycles, sensitive bacteria may become transitorily resistant to infection; **(C) spatial refuges** states that physical heterogeneity in the environment protects some sensitive bacteria from bacteriophage infection; and **(D) shielding by bacterial debris** predicts that active bacteriophages adsorb onto fragments of lysed cells (debris) and inject its genetic material in a suicidal manner discounting from the system as a bacteriophage.

Biological basis of bacteriophage therapy safety

Bacteriophages are considered to have a tropism that is unique to bacteria (60) because adhesion is mediated only by bacterial ligands, and different metabolic pathways preclude bacteriophage replication in human cells. This is well established by molecular biology studies in which bacteriophages that were genetically manipulated to penetrate human cells had no replicative capacity within them (82). However, Merril et al. (83) conducted complicated experiments to demonstrate the possibility of the expression of a bacterial-derived viral gene in human cells. Nevertheless, this is so rare that it does not require serious consideration when accessing the safety of bacteriophages.

The pathogenic potential of bacterial species is caused by the presence and expression of genes that encode for virulence factors. In a process called bacteriophage conversion, bacteriophage-encoded virulence genes can convert their bacterial host from a nonpathogenic strain to a virulent strain by providing mechanisms for the invasion of host tissues and the avoidance of host immune defenses (84). Several temperate bacteriophages have been shown to carry such virulence genes, thus exerting roles in the various stages of pathogenesis: attachment and colonization (85, 86); host immune avoidance (87); cellular invasion (88); intracellular survival (89, 90); and toxin production (91-95). Nonetheless, lateral gene transfer may also occur in strictly lytic bacteriophages by a process known as generalized transduction. Although it should be recognized, this potential biosafety problem should not be overestimated (96) because of the following factors: (1) human commensal bacteria and their respective bacteriophages interact continuously in the body, causing greater lateral gene transfer than the one potentially induced by therapeutic bacteriophages; and (2) it is now generally accepted that antibiotic therapy by itself is a potential inducer (97) and enhancer (98) of lateral gene transfer.

Bacteriophage therapy

Introduction

Bacteriophage therapy (BT) is the use of bacteriophages with the goal of reducing or eliminating pathogenic bacteria. It should be differentiated from bacteriophage biocontrol, which refers to the control of environmental pests or nuisance bacteria by bacteriophages (99). BT holds great promise as a means of controlling infections caused by antibiotic resistant bacteria (independently or synergistically with chemical antibiotics) or under circumstances where chemical antibiotics are otherwise ineffective (100).

Historical perspective

The history of bacteriophage biology and BT (Figure A1-9) has been recounted in some depth and from various perspectives in a number of reviews (101-107). Not long after the discovery of bacteriophages, Felix d'Herelle used them to treat dysentery (59). The first publication on BT was performed by Bruynoghe and Maisin (108), who successfully used bacteriophages in the treatment of *S. aureus* skin infections. Several promising studies followed (109-111), and, encouraged by these results, d'Herelle and others continued their trials in BT. Additionally, in the 1930s, multiple companies (e.g., L'Oreal, Eli Lilly Company, E.R. Squibb & Sons and Swan-Myers-a division of Abbott Laboratories (112)) began the marketing of bacteriophage products that were active against various microorganisms, but not subjected to external evaluations to assess their clinical effectiveness (113).

However, the effectiveness of the preparations was controversial because of the lack of properly controlled studies, quality control, and sufficient understanding of the biology of bacteriophages (112)). Two negative reviews were ordered by the Council on Pharmacy and Chemistry of the US (114, 115). The advent of antibiotics resulted in the cessation of commercial production in much of the Western world by the 1940s. However, in Western Europe, some interest in BT remained and resulted in sporadic publications (116). In France, Dr. Jean-François Vieu of the "Service des Enterobacteries" of the Pasteur Institute of Paris used BT in selected cases during the 1970s (117), and in Vevey, Switzerland, Dr. Glauser Harrmann developed and marketed (with the approval of the National Pharmacy Commission) bacteriophage preparations for therapeutic application (118). Conversely, BT continued to be used, separately or synergistically with antibiotics, in Eastern Europe and the former Soviet Union (119).

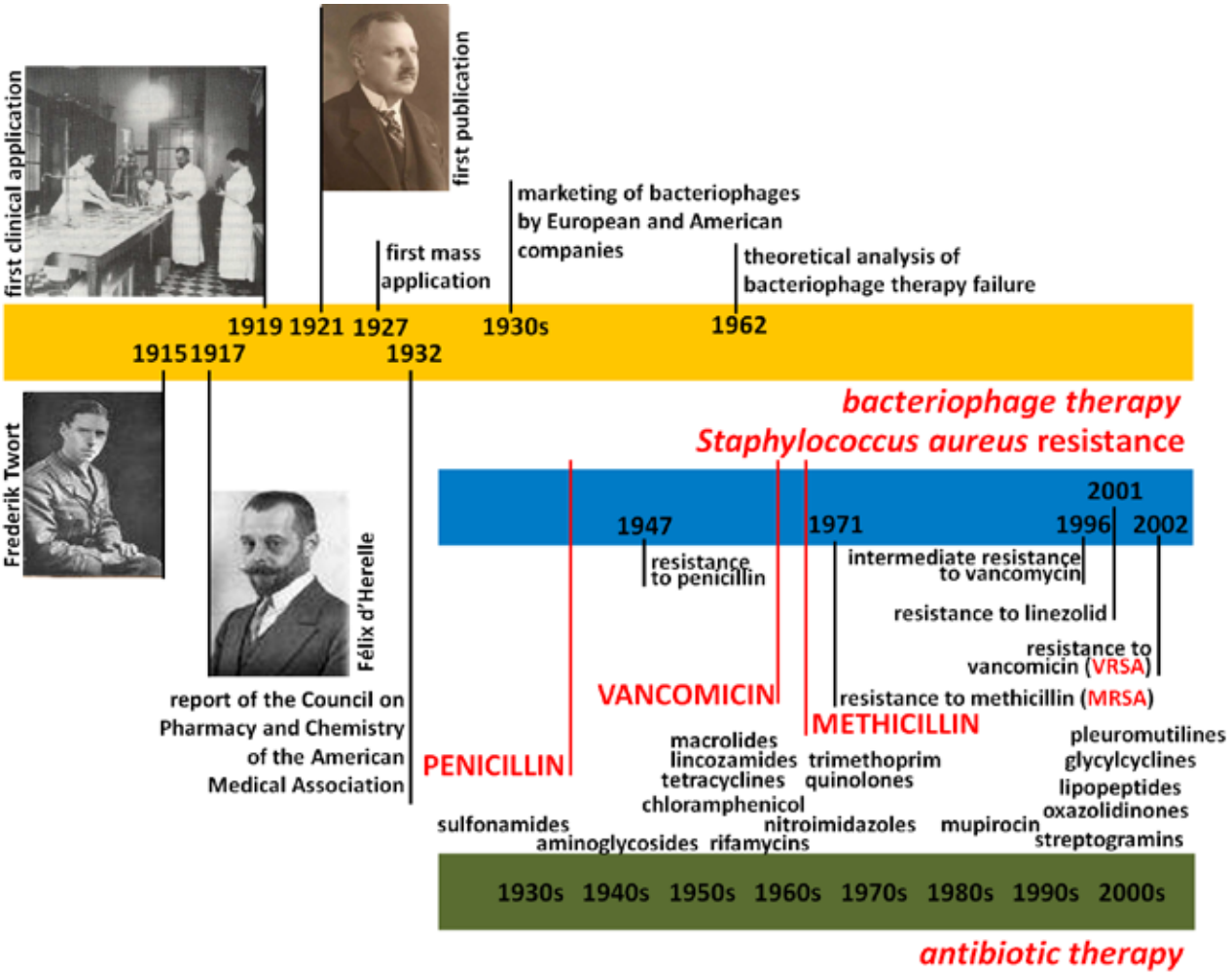


Figure A1-9 – Timeline depicting the comparative evolution of bacteriophage therapy, discovery of antibiotics and the S*taphylococcus aureus* resistance profile, as an illustrative example of the bacterial resistance evolution. MRSA – Methicillin-resistant *Staphylococcus aureus*; VRSA – Vancomycin-resistant *Staphylococcus aureus*

Bacteriophage therapy design

Baceriophage isolation, choice and purification

The first steps in all BT protocols involve some combination of bacteriophage isolation and selection (101, 120, 121). As it is practiced, BT relies on two possible, or even complementary, models of bacteriophage selection (Figure A1-10): large-scale uniform therapy (*prêt-à-porter*) and personalized therapy (*sur-mesure*) (122). The first involves mixtures (cocktails) of different bacteriophages that display a wider host range than their individual components do. The major advantages of this kind of application are the wider spectrum of activity and reduced resistance development in the short term. Hence, along with intellectual property issues, western pharmaceutical companies are pursuing this approach. Thus, the first modern, commercially available human therapeutic bacteriophage preparation will probably be based on this model (101).

In the second approach (personalized therapy), pathogenic bacteria are isolated from a specific patient and tested against a large, ideally well characterized collection of previously isolated bacteriophages. Although this is not compatible with the current licensing processes, the long and expensive regulatory pathways needed for the large-scale uniform therapy approach are forcing non-profit BT centers to opt for a *sur-mesure* concept, while adhering to standards of ethics, safety

and quality control (123). Significantly, evidence (124) has shown that these custom-designed bacteriophage preparations are more effective than the mainstream-production ones are.

Regardless of the bacteriophage model, the procedures by which bacteriophages are isolated from natural samples (e.g., sewage or river water) are similar and involve some form of bacteriophage enrichment (123). This strategy aims to isolate specific bacteriophages that are readily propagated in the laboratory. Isolation and laboratory amplification is followed by a purification procedure (120). While the simplest of bacteriophage purification protocols involves only clarification of lysed cultures via centrifugation or filtration, more stringent purification using ultracentrifugation are followed by a series of filtration and washing/buffer-exchange steps, or various forms of chromatography, which is always required when an invasive bacteriophage application is envisaged (101).

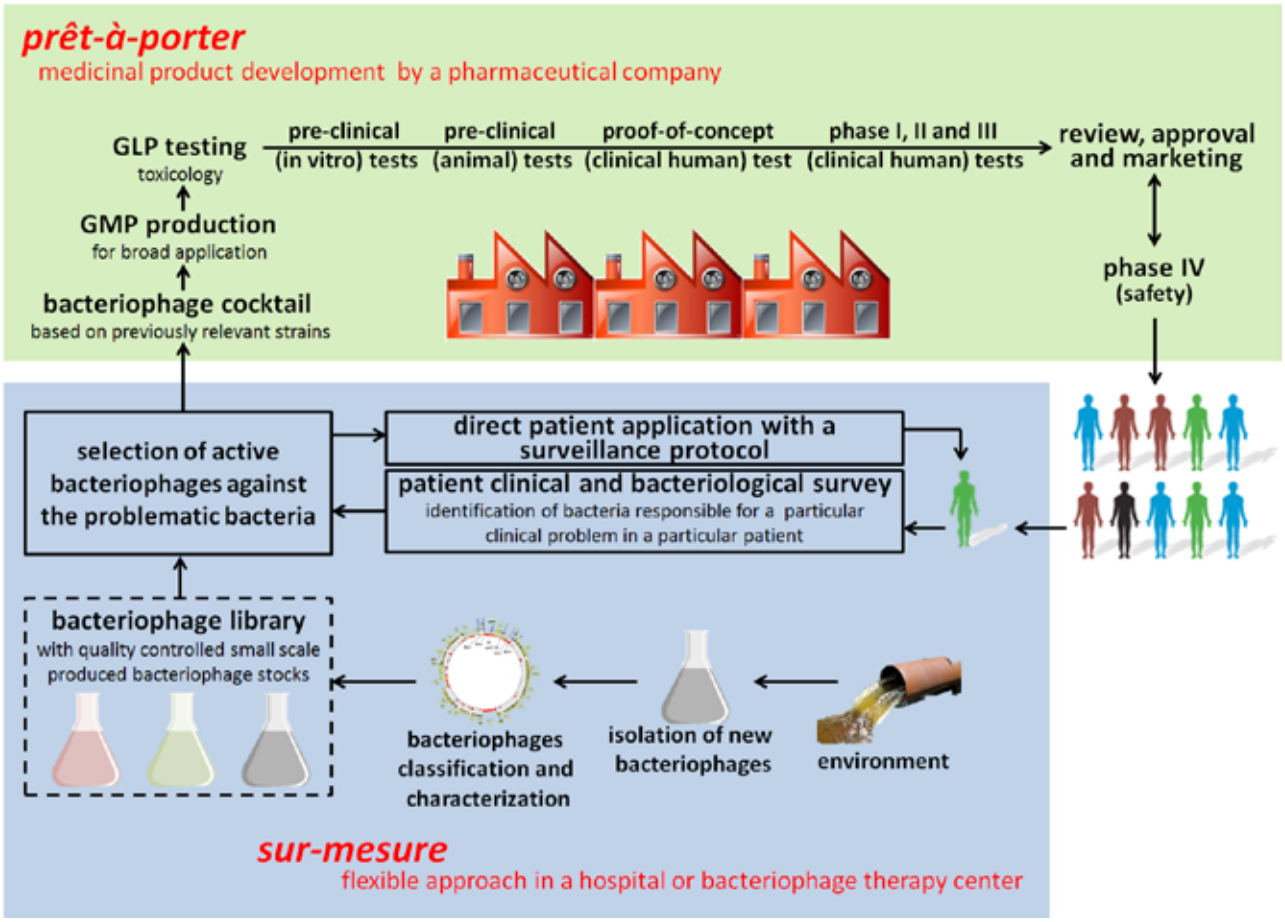


Figure A1-10 – Bacteriophage therapy concepts: *prêt-à-porter* or *sur-mesure* (adapted from (122)). The large-scale uniform therapy (*prêt-à-porter*) model involves mixtures (cocktails) of different bacteriophages that display a wider host range than their individual components do. The major advantages of this kind of application are the wider array spectrum of activity and reduced resistance development in the short term. Hence, along with intellectual property issues, western pharmaceutical companies are following this approach. The personalized therapy (*sur-mesure*) model involves the isolation of pathogenic bacteria from a specific patient and testing against a large, ideally well-characterized collection of previously isolated bacteriophages. While this is not compatible with the current licensing processes, the long and expensive regulatory pathways needed for the large-scale uniform therapy approach are forcing non-profit bacteriophage therapy centers to opt for this concept, while adhering to standards of behavior, safety and quality control.

Pharmacology

Pharmacology may be divided in two major components: pharmacokinetics (description of the body's impact on a drug) and pharmacodynamics (description of a drug's impact on the body). When considering antimicrobials as drugs, the concept of body includes both normal body tissues and the numerous symbiotic microorganisms that constitute the normal microbiota (microbiome), so an important component of antibacterial pharmacodynamics is the inhibition of the growth of target bacteria (66). The pharmacology of BT has been extensively reviewed with emphasis on various aspects of the subject (66, 125, 126).

Bacteriophage application may be topical (applied directly where its action is desired and intending a local effect, including inhalation and enteric non-systemic applications), enteral (given via the digestive tract and intending a systemic effect), and parenteral (given by routes other than the digestive tract and intending a systemic effect) (66). However, this section concentrates on considerations related to topical applications because of its relative importance in this thesis and because it has been relatively under-considered in previous reviews.

Pharmacokinetics

Pharmacokinetics related to topical applications of drugs describes the time-dependent drug concentration following the application of the drug to the surface, which directly relates to its potential to reach concentrations that are sufficient to achieve primary pharmacodynamic effects (127). It also describes the drug's absorption into the systemic circulation and consequent distribution (substance movement into other body tissues), metabolism (modification of drugs within the body) and excretion (movement of drugs out of the body).

We will first focus our pharmacokinetic discussion on a specific bacteriophage characteristic: self-amplification. This characteristic enables not only attaining but also, most importantly, sustaining the minimum bacteriophage density in the vicinity of the target bacteria, which is necessary to achieve the desired levels of eradication (66). The capability of bacteriophages to increase what in pharmacological terms would be their antibacterial dose leads to the concept of passive and active therapy (126, 128, 129). Passive therapy occurs when the initial dose and primary infection is sufficient to reduce bacterial numbers: this can be described as a pharmacologically conventional dosing. On the other hand, active therapy requires the ongoing replication of bacteriophages in order that the bacteriophage density reaches or is maintained at levels sufficient to control the multiplication of bacteria. However, active and passive therapies are not mutually exclusive and both can occur in the same treatment (Figure A1-11). To understand further the basic kinetic properties of BT, we must also appreciate the theoretical concepts of proliferation threshold and inundation threshold (126, 128, 129). The proliferation threshold represents the concentration that the bacterial population must exceed in order for the total bacteriophage numbers to increase. Similarly, the inundation threshold represents the minimum bacteriophage concentration above which the bacterial population declines. By integrating these concepts, we can understand that active therapy can occur only when the concentration of bacteria exceeds the proliferation threshold, and that passive therapy can occur only when the initial concentration of bacteriophage exceeds the inundation threshold. We introduce here the concept of clearance threshold that represents the bacteriophage titer required to reduce the density of a bacterial population to zero (129). Although these concepts are merely theoretical, there is now general consensus that BT protocols should be based on passive therapy using high initial bacteriophage titers approaching the clearance threshold (66, 130). While the attainment of sufficient titers solely via in situ bacteriophage replication (active

therapy) should not be depended upon, bacteriophage replication nonetheless may provide a *margin of safety* in attaining BT efficacy (66). The initial high bacteriophage titers should be calculated based on the multiplicity of the 10 rule (99, 130), which states that if the goal is significant reduction in bacterial density, then we should strive for *on the order* of 10 bacteriophages adsorbed to the average bacterium. Finally, we should stress that bacteriophages display single-hit killing kinetics and that the clearance threshold is calculated based on the number of adsorbed bacteriophages, not the number of added bacteriophages (66).

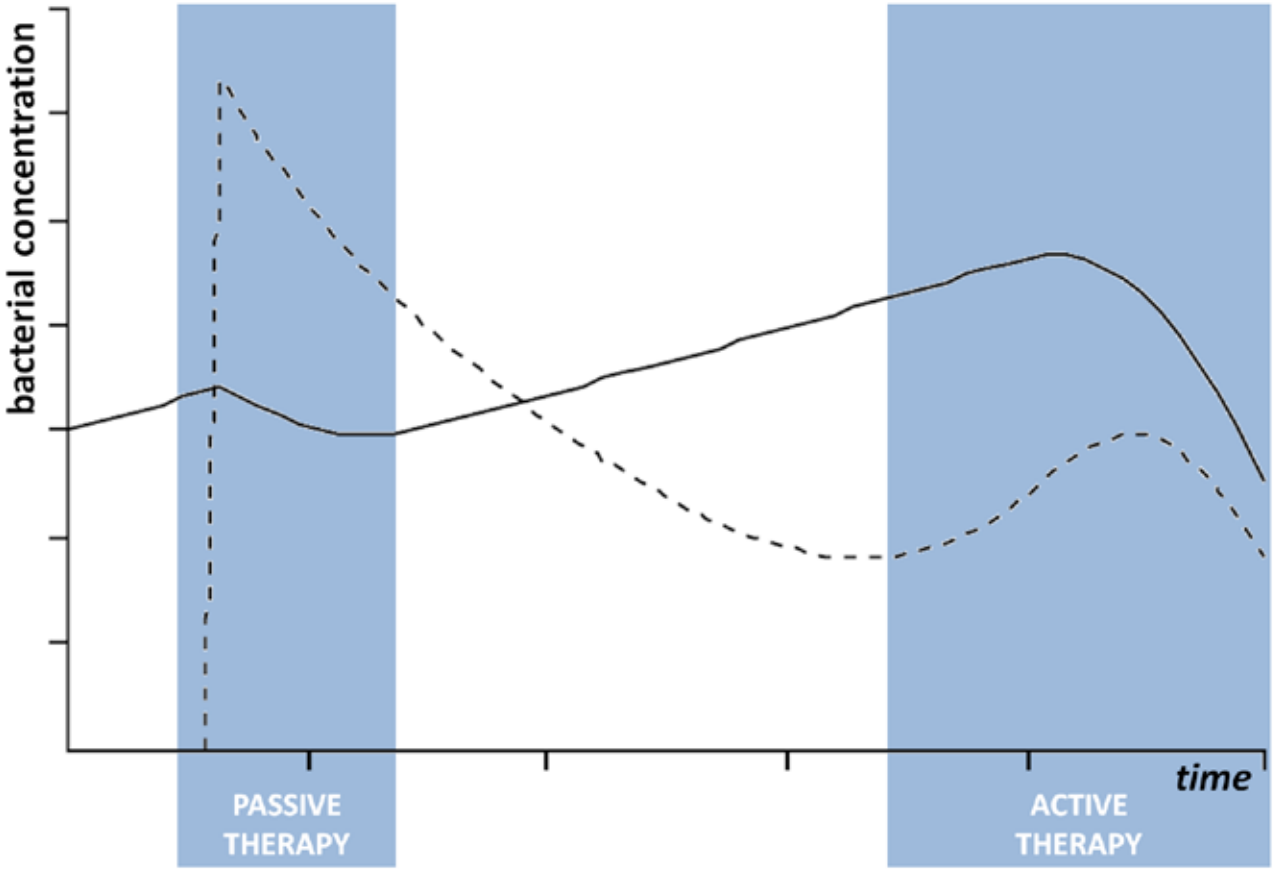


Figure A1-11 – Bacteriophage kinetics (adapted from (129)). Time plot of concentration of bacteria (solid lines) and bacteriophage (broken lines) representing a large inoculation before proliferation onset time, which results in mixed therapy: passive therapy followed by active therapy.

The discussion of absorption, distribution, metabolism, and excretion of bacteriophages following topical application is important mainly in safety considerations. Although several published animal studies concerned the BT of systemic and gastrointestinal infections (131-134), including the evaluation of pharmacokinetic parameters, there is no record of projects dedicated exclusively to topical therapy. However, from these published studies, we can extrapolate some data. A first important point is that even if some studies do not demonstrate systemic absorption of bacteriophages administered by non-parenteral routes (135, 136), bacteriophages applied topically have a propensity to circulate systemically (136-140). After topical application, systemic absorption takes place via the lymphatic system (141) as well as the blood (136), and it is dependent upon the intrinsic characteristics of the bacteriophage, the route of administration, as well as the characteristics of the initial dose, individual species, or host. Few studies have explored the differences among bacteriophages or the differences between individual patients in terms of bacteriophage absorption (66, 125). However, when

absorption occurs, it is very fast and nearly parallel to intravenous administration (142), resulting in the rapid distribution to the internal organs (143). The identification of bacteriophages in the brain, lung, kidney and spleen is possible after 3 hours, although titers are 10^2 lower than in the blood (136, 143). The organs of the mononuclear-phagocyte system (with special relevance to the liver) are the main site of the accumulation of bacteriophages (135, 144), and persistence is only relevant in the spleen (up to 12 days in rabbit (137)) with fast hepatic clearance (145). After administration and systemic absorption, bacteriophages show liver uptake and early excretion in feces (starting at 3 hours and ending 12 hours after adsorption (136, 146)) and urine (beginning 30 minutes after absorption (136)), although this occurs only with blood bacteriophage titers in excess of 10^5 PFU/mL (147). A final consideration is that because of the replicative capacity of bacteriophages, all these characteristics are dependent on the presence or absence of susceptible bacteria and their relative concentration (142), which makes it virtually impossible to design an in vivo pharmacokinetics curve.

Toxicology

Bacteriophages are generally considered safe by the scientific community (148) and regulatory entities (149), based on different theoretical considerations and empirical and experimental evidence. Bacteriophages have been isolated from different human products (saliva (150), urine (151) and feces), constituting a major component of normal gastrointestinal microbiota (142, 152) because of its ability to replicate in symbiotic or pathogenic bacteria. Humans and other higher organisms are often exposed to interactions with bacteriophages, as shown by bacteriophages titers in ground (estimated at 10^7 PFU/g (153)) and aquatic environments (estimated at 10^8 PFU/mL (54)).

Although wastewater treatment plants contain high densities of bacteriophages, most of which have aerosolization potential and are capable of infecting human microbiota bacteria (154-156), no correlation between sewage bacteriophages and human diseases has been identified by public health agencies. Because bacteriophages are normal contaminants of food, it was proposed that the bacterial contamination of certain foods (e.g., carrots (157) and poultry (158)) may be established through counts of specific bacteriophages. Bacteriophages were also identified in commercial preparations of blood products (159, 160) and vaccines (161) for human parenteral administration. Because of concern about the safety of vaccines contaminated with bacteriophages, Milstien et al. (162) isolated and administered them (in high titer) in *Rhesus* monkeys, and did not observe any adverse reaction. Petricciani et al. (163) concluded that human vaccines contaminated with bacteriophages posed no real threat to public health. It is thus possible to conclude that the contact between humans and natural bacteriophages is not incidental but instead intense and constant (142), and that over millions of years, this relationship has never been deleterious. This is also supported by the fact that bacteriophage genes were never identified in the human genome (96), unlike the high level of integration of retroviral genetic material. However, there is the potential for this relationship to change when bacteriophages are applied on a massive scale, as in BT.

The empirical evidence of the safety of bacteriophages is in studies of Western Asia and Eastern Europe countries, which may not meet current standards of clinical research (164), such as Poland (165-171) and Georgia (172), in which no adverse effects were identified with massive exposure (doses between 10^5 and 10^{11}) by oral, rectal, topical and inhaled routes (173). Minor adverse effects (fever and hepatodynia) were identified only in the use of intravenous preparations, and even these were not attributed to bacteriophages, but instead to the bacterial contamination of the byproducts used (174, 175). Perhaps the most complete safety analysis results were found in two studies published by Bogovazova et al. where the pharmacokinetics and toxicology of bacteriophages specific

to *Klebsiella* spp. produced by the Russian firm Immunopreparat™ were analyzed. In the first study (143), bacteriophages administered by intramuscular, intraperitoneal and intravenous injection to rats and guinea pigs resulted in no acute toxicity or macroscopic or histological changes, even at doses 3,500 times higher than those projected for humans. The second study (176) evaluated the safety (and efficacy) of a bacteriophage preparation in the treatment of a range of human patients infected with *Klebsiella* spp., which was considered non-toxic.

The safety of BT was also assured by the experimental evidence in studies meeting the current standards of clinical research, including studies of animal safety (135, 177). Recently, human studies were performed in accordance with experimental patterns in healthy volunteers (178, 179), patients with chronic renal failure (180), patients with congenital immunodeficiencies (179, 181, 182), and patients infected with the human immunodeficiency virus (183).

The recognition of safety is well expressed by authorization granted by the US Food and Drug Administration (FDA) and the US Environmental Protection Agency for the marketing of bacteriophage solutions to control microbial contamination of food: (1) P100 Listex™ to control *Listeria* spp. in meat and cheese (http://www.ebifoodsafety.com) by EBI Food Safety; (2) AgriPhage™ for tomato and pepper harvests by OmniLytics Inc. (http://www.omnilytics.com); and (3) ListShieldTM*,* EcoShieldTM*, or* SalmoFreshTM to control respectively *Listeria* spp., *Escherichia coli* O157:H7 and *Salmonella enterica* by Intralytix Inc. (http://www.intralytix.com). Of special importance is the fact that the Director of the Office of Food Additive Safety, Center for Food Safety and Applied Nutrition of the FDA in the approval declaration of Listex™ P100 (184) concluded that the use of bacteriophage products, either isolated or cumulatively, had no expected significant effects on the human environment, excepting any environmental impact assessment. In addition is Staph Phage Lysate, an immunotherapeutic agent containing high bacteriophage titers (10^8 to 10^9 PFU/mL), which was marketed by Laboratories Delmont for animal use. Its topical and systemic use in human therapy was approved by the National Institute of Health after safety and efficacy studies were conducted in the US (185). The preparation was considered very safe with only minor adverse events reported over a period of 12 years with more than 35,000 SPL doses administered (102). No case of anaphylaxis, even with intravenous therapy, was observed (185).

Efficacy

BT has been extensively studied in animal models used in veterinary medicine (186), and some of the strongest evidence of its efficacy comes from animal studies. The number of papers in the BT field has increased substantially in recent years; however, the number of papers dedicated specifically to the topical application of bacteriophages is limited (187). Because excellent reviews dedicated to animal research studies have been conducted in the past few years (188-190), we will attempt to address the gap in the current knowledge on topical BT by examining the previously published animal studies of the efficacy of using bacteriophages in topical application. For historical interest, we will first refer the studies of Smith et al. (131-134) at the Institute for Animal Disease Research in the United Kingdom, which reported the successful use of bacteriophages in the microbiological and clinical cure of *E. coli* gastrointestinal infections in multiple animal models (mice, calves, lambs, and piglets). These studies constitute the basis of the mathematical models underlying BT presented in the previous section (76, 188), and they impelled other researchers to investigate the effect of BT in different animal models (173). The first relevant animal experimental studies exploring the utility of BT in skin wounds were by Soothill et al. (191, 192), which reported the utility of bacteriophages in preventing and treating experimental disease in skin grafts of rodents infected with *S. aureus* and nonfermenting gram-negative bacilli.

Recently, experimental animal studies have focused on the effective concentrations of bacteriophage needed to eliminate bacterial infections and the timing of administration. A study by Goode et al. (193) used lytic bacteriophages to reduce the contamination of chicken skin by *Salmonella* and *Campylobacter* spp., and a study by Kumari et al. (194) used a specific bacteriophage for the treatment of *K. pneumoniae* B5055-induced burn wound infections. Both studies concluded that low-titer bacteriophage administration is unlikely to be successful, and that increasing the IM increases the success of BT by reducing bacterial numbers. Other experimental animal studies that explored topical BT for areas other than the skin (195, 196) drew similar conclusions.

Some of these previous animal studies have been used to outline the optimal bacteriophage posology and other pertinent details considered important in designing subsequent human volunteer trials. Bogovazova et al. (143) evaluated the efficacy and delineated the optimal bacteriophage concentration and administration route of bacteriophages for the treatment of infections caused by *Klebsiella* spp**.,** and they subsequently used these results to evaluate the safety and efficacy of the bacteriophages in human *Klebsiella* spp. infections (176). Recently, the BioControl company (recently acquired by Targeted Genetics of Seattle to form a new joint company, AmpliPhi Biosciences Corporation) developed a topical bacteriophage formulation for *P. aeruginosa* otitis. The microbiological and clinical efficacy of this formulation was accessed in a canine model (197), and the results were used to develop a double-blinded safety and small-number efficacy trial in human patients suffering chronic otitis caused by *P. aeruginosa*. Finally, we should stress that animal studies conducted to evaluate the efficacy of therapeutic or prophylactic non-topical application of bacteriophages help to corroborate the general efficacy of BT, regardless of the form of application. In this regard, we would like to point out Matsuzaki et al.'s (198) study, which demonstrated the prophylactic effect of bacteriophage ΦMR11 when it was administered before a lethal dose of *S. aureus*. Most importantly, this study included as a negative control a bacterial lysate that did not contain a viable bacteriophage, which did not exert any protective effect, and thus excluded unspecific primer immunogenic effects of some bacteriophage proteins.

Human applications

Excellent reviews of various aspects of human BT are available (100-103, 199-202). Here we will focus on recent relevant studies, as well as Polish and Georgian historical experiences related to the topical BT of wounds. First, because of its historical importance we refer to studies by Slopek et al. (166-171), which constitute the most detailed documentation to date of the general application of bacteriophages for the treatment of human infections. This set of studies involved 550 patients from all age groups in 10 Polish medical centers: the Wroclaw Medical Academy Institute of Surgery Cardiosurgery Clinic, the Children's Surgery Clinic and Orthopedic Clinic, the Institute of Internal Diseases Nephrology Clinic, and the Clinic of Pulmonary Diseases. All treatments were conducted in a research mode, and the bacteriophage formulations were prepared at the Bacteriophage Laboratory of the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, using standard methods and tested for sterility. The treatment protocol included the use of bacteriophage-soaked compresses applied as dictated by localized infection, in addition to systemic (oral) BT. Bacterial levels and bacteriophage sensitivity were continually monitored, and therapy was generally continued for two weeks beyond the last positive bacterial culture. In the final summary paper, the investigators thoroughly analyzed the results and presented success rates ranging from 75% to 100% (92% overall), as measured by wound healing and microbiological improvement. However a more recent analysis, based on the retrospective data of 153 patients

admitted for bacteriophage treatment between January 2008 and December 2010 at the Institute of Immunology and Experimental Medicine (203), showed a lower success rate (39.9%). This was at least partially, justified by more rigorous methods of monitoring and evaluating the patients. Nonetheless, this Institute continues active exploration of different human applications of BT (204-207), with a strong focus on the immunologic consequences of bacteriophage treatment (152, 201, 208, 209).

BT is also part of the general standard of care in Tbilisi, Republic of Georgia, under the auspices of the Bacteriophage Institute. A recent publication by Chanishvili and Sharp (210) drew worldwide attention to the depth and extent of the work developed by this center. The chapter on dermatology reports on the treatment of deep forms of dermatitis, such as furunculosis and abscesses. The protocol included drainage of the abscess and debridement of devitalized tissue followed by injection of the bacteriophage solution into the lesion and surrounding areas, with a reported treatment success of 94.4% in patients who had previously failed antibiotic treatment.

We should stress that currently in the major tertiary care centers of Georgia, BT is a primary tool for the successful treatment of multi-resistant infections. However, the application of bacteriophages is integrated into a broader wound care protocol that includes drainage, debridement, and early wound closure. Bacteriophage can be applied by direct irrigation of the wound with liquid preparations, soaking of bandages in liquid preparations, or by ultrasonic debridement of the wound in the preparation. Preference is generally given to using Pyophage cocktail (a commercial cocktail directed against five different bacterial species, including *P. aeruginosa*), at its standard concentration of 10^5-10^6 pfu/ml of each of the bacteriophage components (100).

Recently, Markoishvili et al. (211) reported on the use of PhagoBioDerm—a biodegradable matrix impregnated with bacteriophages (*S. aureus*, *P. aeruginosa*, *E. coli*, *Streptococcus* spp. and *Proteus* spp.), ciprofloxacin, and benzocaine—to treat infected venous stasis skin ulcers in patients that had failed to respond to other treatment approaches. This study showed promising results, which were later confirmed in another study of radiation burns infected by multiresistant *S. aureus* (212). However, both studies received much criticism because they took place in Georgia, were uncontrolled, and did not yield microbiological evidence in most cases. In 2008, at the Wound Care Center (213) in Lubbock, Texas, a successful, physician-initiated, FDA-approved phase 1 safety trial of BT used a special formulation of fully sequenced bacteriophages prepared by the Intralytix company, containing only two bacteriophages active against *S. aureus*, five against *P. aeruginosa* and one against *E. coli* for the treatment of infected venous ulcers and other chronic wounds. This study managed to demonstrate the safety of the formulation, but it has to be complemented by a phase 2 efficacy study because the posology (dose and dosing interval) used (those permitted by the FDA) did not agree with existing data on bacteriophage pharmacokinetics.

Finally, we refer to the Belgian experience, which explored the possibility of using bacteriophages for human therapy, particularly in burn applications. During this process they developed an extensive collaboration with bacteriophage biologists in both Moscow and Tbilisi. In addition, an international organization, the Phages for Human Applications Group Europe (P.H.A.G.E.), was created for the promotion of research and clinical trials in BT, integrated within a regulated framework (http://www.p-h-a-g-e.org/). As a key step towards full clinical trials of BT, the group carried out a small clinical safety study in burn patients infected with *P. aeruginosa* and/or *S. aureus* at the Brussels Burn Wound Centre of the Queen Astrid Military Hospital in Brussels. This study, which was approved by a medical ethics committee, included 9 patients and was launched following a process

which has been published step-by-step (123, 214). The formulation (BFC-1 bacteriophage cocktail) consisted of three bacteriophages: a *Myovirus* and a *Podovirus* against *P. aeruginosa* and a Myovirus against *S. aureus*. The genome and proteome analysis were consistent with the conclusions that the chosen bacteriophages were not temperate and that there was an absence of toxin-coding genes. The cocktail was purified of endotoxin, and an elaborate quality control was performed including stability (shelf life), determination of pyrogenicity, sterility, and cytotoxicity. In the human study, a section of a large burn on each patient was exposed to a single bacteriophage spray application and another section, distant from the previous and belonging to the same wound, was used as a control. Both regions were evaluated with tissue biopsies before application and between 2 and 5 hours after the treatment application by bacterial quantitative culture. While no adverse events related to the application of bacteriophages were identified, some technical problems were encountered, which limited the evaluation of efficacy. Biopsy samples were found to be excessively cumbersome, leading to long periods between the detection of a candidate with bacterial burn wound colonization and the inclusion of this patient in the study. To address these problems, in the next phase of the trial, patients will be selected based on visual observation of burn wound infection by an experienced clinician, and swabs will be used to monitor burn wound colonization. This will mean the inclusion of all burn wound infections, not just those with *P. aeruginosa* and *S. aureus*. The posology will also be different, with frequent applications (at least once a day) of larger quantities of the bacteriophage formulation in a cream or gel-based product (100). This very active international consortium has now submitted a new study protocol to conduct a prospective randomized double blind trial comparing mupirocin and bacteriophage ISP for the nasal decolonization of MRSA. The complete microbiological and molecular analysis of this therapeutically important bacteriophage, which includes stability assays, genome and virion analysis and an extensive host range screening, has already been published (215).

Regulatory issues

One of the major challenges in the clinical application of bacteriophages in Western medicine is adapting the regulatory framework to fit these very different self-replicating and self-limiting antimicrobials. As described in the previous sections, BT products have already been marketed in European countries. In Poland, which is a member of the European Union, a short term interim solution was to consider BT as an experimental treatment within the responsibility and supervision of medical ethical committees, as covered by the Physician Practice Act (Polish Law Gazette N° 28 of 1997) and World Medical Association Declaration of Helsinki (214, 216). However, this provisional solution is not a substitute for fully controlled clinical trials in accordance with the US FDA or the European Medicines Agency (EMA) regulatory framework.

Recently, EMA placed BT under the Medicinal Product Regulation, specifically under the category of Biologicals (Commission Directive 2001/83/EC). In the US FDA, bacteriophage applications are handled by the Division of Vaccines and Related Product Applications of the Center for Biologicals Evaluation and Research. However, neither of these guidelines fully cover aspects that are specific to bacteriophages. EMA and US FDA might have to revise their rules as they did for influenza vaccines, which also require a rapid updating and licensing procedure (217). These regulatory hurdles, along with the absence of strong intellectual property protection, have hampered pharmaceutical companies in the worldwide marketing of bacteriophage preparations. To avoid the drug licensing pathway, some US-based bacteriophage companies decided to develop bacteriophage products first for the decontamination of food, plants, fields and livestock (218). The US FDA and the US

Department of Agriculture Food Safety and Inspection Service (USDA, FSIS) currently recognize commercial bacteriophage preparations as safe and approved their use in food consumed by humans (71 Fed. Reg. 47729; 2006).

Non-profit organizations, such as P.H.A.G.E., advocate that bacteriophages should not be categorized as classical medicinal products. In the specific case of the European Union, these organizations lobby competent authorities and policymakers for the creation of an optimal regulatory framework dedicated specifically to BT, in addition to the present Medicinal Products Directive 2001/83/EC (219).

Advantages and disadvantages of bacteriophage therapy relative to chemical antibiotics

Lytic bacteriophages have self-replicating and self-limiting antibacterial properties, which makes them compelling alternatives to chemical antibiotics. The majority of the advantages and limitations associated with BT have already been presented in previous sections. Here we will condense them into a coherent whole, especially with regard to topical therapeutic bacteriophage.

As extensively discussed, bacteriophages have a low potential for intrinsic toxicity (119) and, owing to their host specificity, their disruption of normal microbiota tends to be minimal (220). This is in contrast with many chemical antibiotics, which, even when used topically, have unfavorable toxicity profiles and broader spectrums of activity, making them prone to induce superinfections (221). While the narrow spectrum of activity ascribed to bacteriophages is an advantage with regard to safety, it also places a heavy burden on the effective identification of pathogenic bacteria, especially from the point of view of bacteriophage sensitivity (222).

Lytic bacteriophages are bactericidal agents with a specific mechanism of action. Conversely, many antibiotics are bacteriostatic and hence may more readily permit bacterial evolution towards resistance (223). Because antibiotics and bacteriophages as therapeutic agents have different mechanisms of action, there is an absence of cross-resistance. Consequently, BT can be readily employed to treat antibiotic-resistant infections (200, 224).

Bacteriophages are also versatile in terms of formulation development (220), in addition to their suitability for most routes of administration (100, 200). Thereby, one of their major advantages in relation to chemical antibiotics is the feasibility of topical application. Systemic antibiotics may not penetrate sufficiently into the infectious focus to provide sufficient on site concentration to eliminate the infection. This is mainly because of tissue hypoperfusion arising from vascular occlusive diseases, tissue necrosis, or fibrotic barriers. Similarly, antibiotics administrated topically are unable to provide effective wound concentration because of dilution by inflammatory exudates, neutralization by enzymes and other inflammatory mediators, and the inability to penetrate adequately into the necrotic tissues. The reproductive ability of bacteriophages avoids this problem because they continue to replicate and penetrate into tissue as long as susceptible bacteria are present (66, 100). Similarly, it is now recognized that the overwhelming majority of bacteria in chronic wounds exist in a biofilm phenotype (225) and, as previously described, bacteriophages have the potential to penetrate biofilms actively.

Finally, bacteriophages are easily discovered (often from sewage and waste waters) and, especially for external applications, can be prepared fairly inexpensively to facilitate their potential applications, especially in underserved populations (226).

The major disadvantages associated with BT have already been partially addressed and are enumerated as follows: (1) bacteriophages specificity implies that their lytic spectrum may be limited and that causative bacterial pathogens have to be identified prior to their administration; (2) the

lytic life-cycle of bacteriophages described in in vitro environments may not be maintained under normal physiological conditions found in the wound; (3) bacteriophages may have and express genes that encode for virulence factors; (4) resistance to bacteriophage infection may be accomplished by mutation and selection; (5) bacteriophages are complex organisms that may potentially induce a noxious immune response from the host (highly dependent of the on the route of administration and bacteriophage type); and (6) there are limited proofs of efficacy of BT and reported cases of inefficacy. These concerns, specifically those related to safety, should be manageable through a combination of proper bacteriophage selection, effective formulation, and the clinician's understanding of and familiarity with product application (222).

Bacteriophage applications for the treatment of diabetic foot infections

Because antibiotics still represent the standard first-line therapy against bacterial infections, the envisaged use of BT should focus on infections involving antibiotic-resistant bacteria and/or chronic infections in areas not accessible to antibiotic therapy. The role of microorganisms in chronic wounds and the systemic and topical antimicrobial therapy of chronic wounds have been discussed in the previous sections. However, the use of antibiotics is not risk-free, and antimicrobial resistance in the general population is a continuing and growing concern (227). However, a report from the European Centre for Disease Prevention and Control and the EMA pointed out that only two new antibiotic drugs are under development, and both are in the early stages (228). Therefore, infected chronic wounds constitute one of the best field models for the application of BT. Among infected chronic wounds, DFIs are of particular interest for BT because of some intrinsic adverse characteristics, including poor vascularization and the presence of biofilm-associated infections frequently caused by antibiotic-resistant microorganisms (18, 45). In addition, DFIs are a leading cause of morbidity and mortality worldwide, also affecting the working-age population**.** The use of BT in DFI is not unfamiliar in medicine and has been used in both Europe and the United States. However, BT is not part of accepted treatment modalities in the western world, and proof of its efficacy is mainly found in uncontrolled studies (165) and individual case reports (229). Thus, the medical implementation of BT as a fully accepted alternative therapy in DFIs requires the prior pharmacologically informed modeling and development of approaches—in vitro and in vivo—using appropriate animal models in compliance with regulatory frameworks.

References

1. Mustoe TA, O'Shaughnessy K, Kloeters O. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. Plast Reconstr Surg. 2006 Jun;117(7 Suppl):35S-41S.
2. Posnett J, Franks PJ. The burden of chronic wounds in the UK. Nurs Times. 2008 Jan 22-28;104(3):44-5.
3. Thomas S. Cost of managing chronic wounds in the U.K., with particular emphasis on maggot debridement therapy. J Wound Care. 2006 Nov;15(10):465-9.
4. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999 Sep 2;341(10):738-46.
5. Schreml S, Szeimies RM, Prantl L, Landthaler M, Babilas P. Wound healing in the 21st century. J Am Acad Dermatol. 2010 Nov;63(5):866-81.
6. Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecoraro RE, Rodeheaver G, et al. Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol. 1994 Apr;130(4):489-93.
7. Wolcott RD, Rhoads DD, Bennett ME, Wolcott BM, Gogokhia L, Costerton JW, et al. Chronic wounds and the medical biofilm paradigm. J Wound Care. 2010 Feb;19(2):45-6, 8-50, 2-3.
8. Pierce GF. Inflammation in nonhealing diabetic wounds: the space-time continuum does matter. Am J Pathol. 2001 Aug;159(2):399-403.
9. Jeffcoate WJ, Price P, Harding KG. Wound healing and treatments for people with diabetic foot ulcers. Diabetes Metab Res Rev. 2004 May-Jun;20 Suppl 1:S78-89.
10. Howell-Jones RS, Wilson MJ, Hill KE, Howard AJ, Price PE, Thomas DW. A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. J Antimicrob Chemother. 2005 Feb;55(2):143-9.
11. Lipsky BA. Evidence-based antibiotic therapy of diabetic foot infections. FEMS Immunol Med Microbiol. 1999 Dec;26(3-4):267-76.
12. Gerding DN. Foot infections in diabetic patients: the role of anaerobes. Clin Infect Dis. 1995 Jun;20 Suppl 2:S283-8.
13. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001 Apr;14(2):244-69.
14. Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen. 2008 Jan-Feb;16(1):23-9.
15. Nakagami G, Morohoshi T, Ikeda T, Ohta Y, Sagara H, Huang L, et al. Contribution of quorum sensing to the virulence of Pseudomonas aeruginosa in pressure ulcer infection in rats. Wound Repair Regen. 2011 Mar-Apr;19(2):214-22.
16. Breidenbach WC, Trager S. Quantitative culture technique and infection in complex wounds of the extremities closed with free flaps. Plast Reconstr Surg. 1995 Apr;95(5):860-5.
17. Dow G, Browne A, Sibbald RG. Infection in chronic wounds: controversies in diagnosis and treatment. Ostomy Wound Manage. 1999 Aug;45(8):23-7, 9-40; quiz 1-2.
18. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. Clin Infect Dis. 2004 Oct 1;39(7):885-910.
19. Kingsley A. The wound infection continuum and its application to clinical practice. Ostomy Wound Manage. 2003 Jul;49(7A Suppl):1-7.
20. Werdin F, Tennenhaus M, Schaller HE, Rennekampff HO. Evidence-based management strategies for treatment of chronic wounds. Eplasty. 2009;9:e19.

21. Armstrong DG, Lavery LA, Vazquez JR, Nixon BP, Boulton AJ. How and why to surgically debride neuropathic diabetic foot wounds. J Am Podiatr Med Assoc. 2002 Jul-Aug;92(7):402-4.
22. Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, et al. Biofilm maturity studies indicate sharp debridement opens a time- dependent therapeutic window. J Wound Care. 2010 Aug;19(8):320-8.
23. Wolcott RD, Kennedy JP, Dowd SE. Regular debridement is the main tool for maintaining a healthy wound bed in most chronic wounds. J Wound Care. 2009 Feb;18(2):54-6.
24. Steed DL, Donohoe D, Webster MW, Lindsley L. Effect of extensive debridement and treatment on the healing of diabetic foot ulcers. Diabetic Ulcer Study Group. J Am Coll Surg. 1996 Jul;183(1):61-4.
25. Sibbald RG, Williamson D, Orsted HL, Campbell K, Keast D, Krasner D, et al. Preparing the wound bed--debridement, bacterial balance, and moisture balance. Ostomy Wound Manage. 2000 Nov;46(11):14-22, 4-8, 30-5; quiz 6-7.
26. Diehr S, Hamp A, Jamieson B, Mendoza M. Clinical inquiries. Do topical antibiotics improve wound healing? J Fam Pract. 2007 Feb;56(2):140-4.
27. O'Meara S, Cullum N, Majid M, Sheldon T. Systematic reviews of wound care management: (3) antimicrobial agents for chronic wounds; (4) diabetic foot ulceration. Health Technol Assess. 2000;4(21):1-237.
28. Lipsky BA, Hoey C. Topical antimicrobial therapy for treating chronic wounds. Clin Infect Dis. 2009 Nov 15;49(10):1541-9.
29. O'Meara SM, Cullum NA, Majid M, Sheldon TA. Systematic review of antimicrobial agents used for chronic wounds. Br J Surg. 2001 Jan;88(1):4-21.
30. O'Meara S, Al-Kurdi D, Ologun Y, Ovington LG. Antibiotics and antiseptics for venous leg ulcers. Cochrane Database Syst Rev. 2010(1):CD003557.
31. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004 May;27(5):1047-53.
32. Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. JAMA. 2005 Jan 12;293(2):217-28.
33. Prompers L, Huijberts M, Apelqvist J, Jude E, Piaggesi A, Bakker K, et al. Optimal organization of health care in diabetic foot disease: introduction to the Eurodiale study. Int J Low Extrem Wounds. 2007 Mar;6(1):11-7.
34. Jeffcoate WJ, Harding KG. Diabetic foot ulcers. Lancet. 2003 May 3;361(9368):1545-51.
35. Levin ME. Foot lesions in patients with diabetes mellitus. Endocrinol Metab Clin North Am. 1996 Jun;25(2):447-62.
36. Boulton AJ, Meneses P, Ennis WJ. Diabetic foot ulcers: A framework for prevention and care. Wound Repair Regen. 1999 Jan-Feb;7(1):7-16.
37. Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA. Risk factors for foot infections in individuals with diabetes. Diabetes Care. 2006 Jun;29(6):1288-93.
38. Hoogwerf BJ, Sferra J, Donley BG. Diabetes mellitus-overview. Foot Ankle Clin. 2006 Dec;11(4):703-15.
39. Apelqvist J, Bakker K, van Houtum WH, Schaper NC. Practical guidelines on the management and prevention of the diabetic foot: based upon the International Consensus on the Diabetic Foot (2007) Prepared by the International Working Group on the Diabetic Foot. Diabetes Metab Res Rev. 2008 May-Jun;24 Suppl 1:S181-7.

40. Reiber GE, Vileikyte L, Boyko EJ, del Aguila M, Smith DG, Lavery LA, et al. Causal pathways for incident lower-extremity ulcers in patients with diabetes from two settings. Diabetes Care. 1999 Jan;22(1):157-62.
41. Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus (DM). FEMS Immunol Med Microbiol. 1999 Dec;26(3-4):259-65.
42. Hartemann-Heurtier A, Robert J, Jacqueminet S, Ha Van G, Golmard JL, Jarlier V, et al. Diabetic foot ulcer and multidrug-resistant organisms: risk factors and impact. Diabet Med. 2004 Jul;21(7):710-5.
43. Schaper NC. Diabetic foot ulcer classification system for research purposes: a progress report on criteria for including patients in research studies. Diabetes Metab Res Rev. 2004 May-Jun;20 Suppl 1:S90-5.
44. Widatalla AH, Mahadi SE, Shawer MA, Elsayem HA, Ahmed ME. Implementation of diabetic foot ulcer classification system for research purposes to predict lower extremity amputation. Int J Diabetes Dev Ctries. 2009 Jan;29(1):1-5.
45. Mendes JJ, Neves J. Diabetic foot infections: current diagnosis and treatment. The Journal of Diabetic Foot Complications. 2012;4(2):26-45.
46. Frykberg RG. Diabetic foot ulcers: current concepts. J Foot Ankle Surg. 1998 Sep-Oct;37(5):440-6.
47. Berendt AR, Peters EJ, Bakker K, Embil JM, Eneroth M, Hinchliffe RJ, et al. Diabetic foot osteomyelitis: a progress report on diagnosis and a systematic review of treatment. Diabetes Metab Res Rev. 2008 May-Jun;24 Suppl 1:S145-61.
48. Lavery LA, Armstrong DG, Peters EJ, Lipsky BA. Probe-to-bone test for diagnosing diabetic foot osteomyelitis: reliable or relic? Diabetes Care. 2007 Feb;30(2):270-4.
49. Bonham PA. Swab cultures for diagnosing wound infections: a literature review and clinical guideline. J Wound Ostomy Continence Nurs. 2009 Jul-Aug;36(4):389-95.
50. National Institute for Health and Clinical Excellence. NICE clinical guideline 119: Inpatient management of diabetic foot problems. London, UK: National Institute for Health and Clinical Excellence; 2011.
51. Lipsky BA, Stoutenburgh U. Daptomycin for treating infected diabetic foot ulcers: evidence from a randomized, controlled trial comparing daptomycin with vancomycin or semi-synthetic penicillins for complicated skin and skin-structure infections. J Antimicrob Chemother. 2005 Feb;55(2):240-5.
52. Nelson EA, O'Meara S, Golder S, Dalton J, Craig D, Iglesias C. Systematic review of antimicrobial treatments for diabetic foot ulcers. Diabet Med. 2006 Apr;23(4):348-59.
53. Adam DJ, Beard JD, Cleveland T, Bell J, Bradbury AW, Forbes JF, et al. Bypass versus angioplasty in severe ischaemia of the leg (BASIL): multicentre, randomised controlled trial. Lancet. 2005 Dec 3;366(9501):1925-34.
54. Bergh O, Borsheim KY, Bratbak G, Heldal M. High abundance of viruses found in aquatic environments. Nature. 1989 Aug 10;340(6233):467-8.
55. Abedon ST. Phage evolution and ecology. Adv Appl Microbiol. 2009;67:1-45.
56. Twort F. An investigation on the nature of ultra-microscopic viruses. Lancet. 1915;2:1241-3.
57. d'Herelle F. An invisible antagonist microbe of dysentery bacillus. Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences. 1917;165:373-5.
58. d'Herelle F. The bacteriophage: its role in immunity. 1st ed. Baltimore, USA: Lippincott Williams & Wilkins; 1922.

59. d'Herelle F. [Les pérégrinations d'un bactériologiste]. Paris, France: Pasteur Institute (Fond Félix d'Herelle); 1921.
60. Raoult D, Forterre P. Redefining viruses: lessons from Mimivirus. Nat Rev Microbiol. 2008 Apr;6(4):315-9.
61. Ackermann HW. Phage classification and characterization. Methods Mol Biol. 2009;501:127-40.
62. Lawrence JG, Hatfull GF, Hendrix RW. Imbroglios of viral taxonomy: genetic exchange and failings of phenetic approaches. J Bacteriol. 2002 Sep;184(17):4891-905.
63. Nelson D. Phage taxonomy: we agree to disagree. J Bacteriol. 2004 Nov;186(21):7029-31.
64. Ackermann HW. Tailed bacteriophages: the order caudovirales. Adv Virus Res. 1998;51:135-201.
65. Abedon ST. Lysis of lysis-inhibited bacteriophage T4-infected cells. J Bacteriol. 1992 Dec;174(24):8073-80.
66. Abedon ST, Thomas-Abedon C. Phage therapy pharmacology. Curr Pharm Biotechnol. 2010 Jan;11(1):28-47.
67. Guttman B, Raya R, Kutter E. Basic phage biology. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: biology and applications. 1st ed. Boca Raton, USA: CRC Press; 2005. p. 29-66.
68. Zoon KC, Habersat M, Scocca JJ. Synthesis of envelope polypeptides by Haemophilus influenzae during development of competence for genetic transformation. J Bacteriol. 1976 Jul;127(1):545-54.
69. Young R. Bacteriophage lysis: mechanism and regulation. Microbiol Rev. 1992 Sep;56(3):430-81.
70. Abedon ST. Bacteriophages and biofilms. In: Bailey WC, editor. Biofilms: formation, development and properties. 1st ed. New York, USA: Nova Science Publishers; 2011. p. 1-58.
71. Sutherland IW, Hughes KA, Skillman LC, Tait K. The interaction of phage and biofilms. FEMS Microbiol Lett. 2004 Mar 12;232(1):1-6.
72. Skraber S, Schijven J, Gantzer C, de Roda Husman AM. Pathogenic viruses in drinking-water biofilms: a public health risk? Biofilms. 2005;2(02):105-17.
73. Azeredo J, Sutherland IW. The use of phages for the removal of infectious biofilms. Curr Pharm Biotechnol. 2008 Aug;9(4):261-6.
74. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol. 2010 May;8(5):317-27.
75. Drulis-Kawa Z, Majkowska-Skrobek G, Maciejewska B, Delattre AS, Lavigne R. Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. Curr Protein Pept Sci. 2012 Dec;13(8):699-722.
76. Levin BR, Bull JJ. Population and evolutionary dynamics of phage therapy. Nat Rev Microbiol. 2004 Feb;2(2):166-73.
77. Bohannan BJ, Lenski RE. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophages. Ecol Lett. 2000;3(4):362-77.
78. Chao L, Levin BR, Stewart FM. A complex community in a simple habitat: an experimental study with bacteria and phage. Ecology. 1977;58:369-78.
79. Lenski RE, Levin BR. Constraints on the coevolution of bacteria and virulent phage: a model, some experiments and predictions for natural communities. Amer Naturalist. 1985 125(4):585-602.
80. Schrag J, Mittler E. Host-parasite coexistence: the role of spatial refuges in stabilizing bacteria-phage interactions. Amer Naturalist. 1996;148(2):348-77.

81. Rabinovitch A, Aviram I, Zaritsky A. Bacterial debris-an ecological mechanism for coexistence of bacteria and their viruses. J Theor Biol. 2003 Oct 7;224(3):377-83.
82. Di Giovine M, Salone B, Martina Y, Amati V, Zambruno G, Cundari E, et al. Binding properties, cell delivery, and gene transfer of adenoviral penton base displaying bacteriophage. Virology. 2001 Mar 30;282(1):102-12.
83. Merril CR, Geier MR, Petricciani JC. Bacterial virus gene expression in human cells. Nature. 1971 Oct 8;233(5319):398-400.
84. Boyd EF. Bacteriophages and bacterial virulence. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: biology and applications. 1st ed. Boca Raton, USA: CRC Press; 2005. p. 223-66.
85. Vica Pacheco S, Garcia Gonzalez O, Paniagua Contreras GL. The lom gene of bacteriophage lambda is involved in Escherichia coli K12 adhesion to human buccal epithelial cells. FEMS Microbiol Lett. 1997 Nov 1;156(1):129-32.
86. Karaolis DK, Somara S, Maneval DR, Jr., Johnson JA, Kaper JB. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature. 1999 May 27;399(6734):375-9.
87. Masignani V, Giuliani MM, Tettelin H, Comanducci M, Rappuoli R, Scarlato V. Mu-like Prophage in serogroup B Neisseria meningitidis coding for surface-exposed antigens. Infect Immun. 2001 Apr;69(4):2580-8.
88. Hynes WL, Ferretti JJ. Sequence analysis and expression in Escherichia coli of the hyaluronidase gene of Streptococcus pyogenes bacteriophage H4489A. Infect Immun. 1989 Feb;57(2):533-9.
89. Barondess JJ, Beckwith J. A bacterial virulence determinant encoded by lysogenic coliphage lambda. Nature. 1990 Aug 30;346(6287):871-4.
90. Ohnishi M, Kurokawa K, Hayashi T. Diversification of Escherichia coli genomes: are bacteriophages the major contributors? Trends Microbiol. 2001 Oct;9(10):481-5.
91. Freeman VJ. Studies on the virulence of bacteriophage-infected strains of Corynebacterium diphtheriae. J Bacteriol. 1951 Jun;61(6):675-88.
92. O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Formal SB. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science. 1984 Nov 9;226(4675):694-6.
93. Weeks CR, Ferretti JJ. The gene for type A streptococcal exotoxin (erythrogenic toxin) is located in bacteriophage T12. Infect Immun. 1984 Nov;46(2):531-6.
94. Betley MJ, Mekalanos JJ. Staphylococcal enterotoxin A is encoded by phage. Science. 1985 Jul 12;229(4709):185-7.
95. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996 Jun 28;272(5270):1910-4.
96. Vaneechoutte M, Merabishvili M, Verbeken G, de Vos D, Pirnay J. Bacteriophages. In: Ministerie van Landbouw NeV, editor. [Alternatieven voor antibiotica]. 1st ed. Den Haag, Netherland: Ministerie van Landbouw, Natuur en Voedselkwaliteit; 2009. p. 55-71.
97. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP. Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. Science. 2006 Jul 7;313(5783):89-92.
98. Kolar M, Urbanek K, Latal T. Antibiotic selective pressure and development of bacterial resistance. Int J Antimicrob Agents. 2001 May;17(5):357-63.
99. Abedon ST. Kinetics of phage-mediated biocontrol of bacteria. Foodborne Pathog Dis. 2009 Sep;6(7):807-15.

100. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, et al. Phage therapy in clinical practice: treatment of human infections. Curr Pharm Biotechnol. 2010 Jan;11(1):69-86.
101. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011 Mar;1(2):66-85.
102. Sulakvelidze A, Kutter E. Bacteriophage therapy in humans. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: biology and applications. 1st ed. Boca Raton, USA: CRC Press; 2005. p. 381-436.
103. Kutter E. Bacteriophage therapy: past and present. In: Schaecter M, editor. Encyclopedia of microbiology. 3rd ed. Oxford, UK: Elsevier; 2009. p. 258-66.
104. Summers WC. History of phage research and phage therapy. In: Waldor M, Friedman D, Adhya S, editors. Phages: Their role in bacterial pathogenesis and biotechnology. 1st ed. Washington DC, USA: ASM Press; 2005. p. 1-17.
105. Summers WC. In the beginning. Bacteriophage. 2011 Jan;1(1):50-1.
106. Summers WC. Bacteriophage research: early research. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: biology and applications. 1st ed. Boca Raton, USA: CRC Press; 2005. p. 5-28.
107. Summers WC. Bacteriophage therapy. Annu Rev Microbiol. 2001;55:437-51.
108. Bruynoghe R, Maisin J. [Essais de thérapeutique au moyen du bacteriophage]. C R Soc Biol. 1921;85:1120-1.
109. Rice TB. Use of bacteriophages filtrates in treatment of suppurative conditions: report of 300 cases Am J Med Sci. 1930;179:345-60.
110. Schless RA. Staphylococcus aureus meningitis: treatmente with specific bacteriophage. Am J Dis Child. 1932;44:813-22.
111. Stout BF. Bacteriophage therapy. Texas State J Med. 1933;29:205-9.
112. Summers WC. Félix d'Herelle and the origins of molecular biology. 1st ed. New Haven, USA: Yale University Press; 1999.
113. Straub ME, Applebaum M. Studies on commercial bacteriophage products. JAMA. 1933;100:110-3.
114. Eaton MD, Bayne-Jones S. Bacteriophage therapy. Review of the principles and results of the use of bacteriophages in the treatment of infections. JAMA. 1934;23:1769-939.
115. Krueger AP, Scribner EJ. Bacteriophage therapy. II. The bacteriophage use and its therapeutic use. JAMA. 1941;19:2160-277.
116. Lang G, Kehr P, Mathevon H, Clavert JM, Sejourne P, Pointu J. [Bacteriophage therapy of septic complications of orthopaedic surgery (author's transl)]. Rev Chir Orthop Reparatrice Appar Mot. 1979 Jan-Feb;65(1):33-7.
117. Vieu JF, Guillermet F, Minck R, Nicolle P. [New trends in therapeutic use of bacteriophages]. Bull Acad Natl Med. 1979 Jan;163(1):61-6; discussion 6.
118. Häusler T. [Gesund durch viren]. 1st ed. Munich, Germany: Piper; 2003.
119. Carlton RM. Phage therapy: past history and future prospects. Arch Immunol Ther Exp (Warsz). 1999;47(5):267-74.
120. Gill JJ, Hyman P. Phage choice, isolation, and preparation for phage therapy. Curr Pharm Biotechnol. 2010 Jan;11(1):2-14.
121. Goodridge LD. Designing phage therapeutics. Curr Pharm Biotechnol. 2010 Jan;11(1):15-27.
122. Pirnay JP, De Vos D, Verbeken G, Merabishvili M, Chanishvili N, Vaneechoutte M, et al. The phage therapy paradigm: pret-a-porter or sur-mesure? Pharm Res. 2011 Apr;28(4):934-7.

123. Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Tediashvili M, Lashkhi N, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One. 2009;4(3):e4944.
124. Zhukov-Verezhnikov NN, Peremitina LD, Berillo EA, Komissarov VP, Bardymov VM. [Therapeutic effect of bacteriophage preparations in the complex treatment of suppurative surgical diseases]. Sov Med. 1978 Dec(12):64-6.
125. Gorski A, Wazna E, Dabrowska BW, Dabrowska K, Switala-Jelen K, Miedzybrodzki R. Bacteriophage translocation. FEMS Immunol Med Microbiol. 2006 Apr;46(3):313-9.
126. Payne RJ, Jansen VA. Pharmacokinetic principles of bacteriophage therapy. Clin Pharmacokinet. 2003;42(4):315-25.
127. Schaefer H, Redelmeier TE, Nohynek GJ. Pharmacokinetics and topical applications of drugs. In: Freedberg IM, editor. Fitzpatrick's Dermatology In General Medicine. 6th ed. New York: McGraw-Hill Professional; 2003.
128. Cairns BJ, Timms AR, Jansen VA, Connerton IF, Payne RJ. Quantitative models of in vitro bacteriophage-host dynamics and their application to phage therapy. PLoS Pathog. 2009 Jan;5(1):e1000253.
129. Payne RJ, Phil D, Jansen VA. Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. Clin Pharmacol Ther. 2000 Sep;68(3):225-30.
130. Kasman LM, Kasman A, Westwater C, Dolan J, Schmidt MG, Norris JS. Overcoming the phage replication threshold: a mathematical model with implications for phage therapy. J Virol. 2002 Jun;76(11):5557-64.
131. Smith HW, Huggins MB. Effectiveness of phages in treating experimental Escherichia coli diarrhoea in calves, piglets and lambs. J Gen Microbiol. 1983 Aug;129(8):2659-75.
132. Smith HW, Huggins MB, Shaw KM. The control of experimental Escherichia coli diarrhoea in calves by means of bacteriophages. J Gen Microbiol. 1987 May;133(5):1111-26.
133. Smith HW, Huggins MB, Shaw KM. Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. J Gen Microbiol. 1987 May;133(5):1127-35.
134. Smith HW, Huggins MB. Successful treatment of experimental Escherichia coli infections in mice using phage: its general superiority over antibiotics. J Gen Microbiol. 1982 Feb;128(2):307-18.
135. Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, et al. Long-circulating bacteriophage as antibacterial agents. Proc Natl Acad Sci U S A. 1996 Apr 16;93(8):3188-92.
136. Keller R, Engley FB, Jr. Fate of bacteriophage particles introduced into mice by various routes. Proc Soc Exp Biol Med. 1958 Jul;98(3):577-80.
137. Reynaud A, Cloastre L, Bernard J, Laveran H, Ackermann HW, Licois D, et al. Characteristics and diffusion in the rabbit of a phage for Escherichia coli 0103. Attempts to use this phage for therapy. Vet Microbiol. 1992 Feb;30(2-3):203-12.
138. Weber-Dabrowska B, Dabrowski M, Slopek S. Studies on bacteriophage penetration in patients subjected to phage therapy. Arch Immunol Ther Exp (Warsz). 1987;35(5):563-8.
139. Hoffmann M. [Animal experiments on the mucosal passage and absorption viremia of T3 phages after oral, tracheal and rectal administration]. Zentralbl Bakteriol Orig. 1965 Dec;198(4):371-90.
140. Georgakopoulos PA. [Permeability of the female genital system to virus]. Arch Gynakol. 1968;205(3):211-8.
141. Hildebrand GJ, Wolochow H. Translocation of bacteriophage across the intestinal wall of the rat. Proc Soc Exp Biol Med. 1962 Jan;109:183-5.

142. Dabrowska K, Switala-Jelen K, Opolski A, Weber-Dabrowska B, Gorski A. Bacteriophage penetration in vertebrates. J Appl Microbiol. 2005;98(1):7-13.
143. Bogovazova GG, Voroshilova NN, Bondarenko VM. [The efficacy of Klebsiella pneumoniae bacteriophage in the therapy of experimental Klebsiella infection]. Zh Mikrobiol Epidemiol Immunobiol. 1991 Apr(4):5-8.
144. Geier MR, Trigg ME, Merril CR. Fate of bacteriophage lambda in non-immune germ-free mice. Nature. 1973 Nov 23;246(5430):221-3.
145. Inchley CJ. The actvity of mouse Kupffer cells following intravenous injection of T4 bacteriophage. Clin Exp Immunol. 1969 Jul;5(1):173-87.
146. Nakai T, Park SC. Bacteriophage therapy of infectious diseases in aquaculture. Res Microbiol. 2002 Jan-Feb;153(1):13-8.
147. Schultz I, Neva FA. Relationship between Blood Clearance and Viruria after Intravenous Injection of Mice and Rats with Bacteriophage and Polioviruses. J Immunol. 1965 Jun;94:833-41.
148. Inal JM. Phage therapy: a reappraisal of bacteriophages as antibiotics. Arch Immunol Ther Exp (Warsz). 2003;51(4):237-44.
149. Food and Drug Administration. GRAS Notice No. GRN 000218: Available from: http://www.cfsan.fda.gov/~rdb/opa-g218.html.
150. Bachrach G, Leizerovici-Zigmond M, Zlotkin A, Naor R, Steinberg D. Bacteriophage isolation from human saliva. Lett Appl Microbiol. 2003;36(1):50-3.
151. Caldwell JA. Bacteriologic and bacteriophagic study of infected urines. J Infect Dis. 1928;43:353-62.
152. Gorski A, Dabrowska K, Switala-Jelen K, Nowaczyk M, Weber-Dabrowska B, Boratynski J, et al. New insights into the possible role of bacteriophages in host defense and disease. Med Immunol. 2003 Feb 14;2(1):2.
153. Ashelford KE, Day MJ, Fry JC. Elevated abundance of bacteriophage infecting bacteria in soil. Appl Environ Microbiol. 2003 Jan;69(1):285-9.
154. Bausum HT, Schaub SA, Kenyon KF, Small MJ. Comparison of coliphage and bacterial aerosols at a wastewater spray irrigation site. Appl Environ Microbiol. 1982 Jan;43(1):28-38.
155. Brenner KP, Scarpino PV, Clark CS. Animal viruses, coliphages, and bacteria in aerosols and wastewater at a spray irrigation site. Appl Environ Microbiol. 1988 Feb;54(2):409-15.
156. Carducci A, Arrighi S, Ruschi A. Detection of coliphages and enteroviruses in sewage and aerosol from an activated sludge wastewater treatment plant. Lett Appl Microbiol. 1995 Sep;21(3):207-9.
157. Endley S, Lu L, Vega E, Hume ME, Pillai SD. Male-specific coliphages as an additional fecal contamination indicator for screening fresh carrots. J Food Prot. 2003 Jan;66(1):88-93.
158. Hsu FC, Shieh YS, Sobsey MD. Enteric bacteriophages as potential fecal indicators in ground beef and poultry meat. J Food Prot. 2002 Jan;65(1):93-9.
159. Merril CR, Friedman TB, Attallah AF, Geier MR, Krell K, Yarkin R. Isolation of bacteriophages from commercial sera. In Vitro. 1972 Sep-Oct;8(2):91-3.
160. Trefouel J. [Bacteriophages contaminating preparations of calf serum for tissue culture]. C R Acad Sci Hebd Seances Acad Sci D. 1974 Aug 12;279(7):615-8.
161. Moody EE, Trousdale MD, Jorgensen JH, Shelokov A. Bacteriophages and endotoxin in licensed live-virus vaccines. J Infect Dis. 1975 May;131(5):588-91.
162. Milstien JB, Walker JR, Petricciani JC. Bacteriophages in live virus vaccines: lack of evidence for effects on the genome of rhesus monkeys. Science. 1977 Jul 29;197(4302):469-70.

163. Petricciani JC, Hsu TC, Stock AD, Turner JH, Wenger SL, Elisberg BL. Bacteriophages, vaccines, and people: an assessment of risk. Proc Soc Exp Biol Med. 1978 Jul;158(3):378-82.
164. Borysowski J, Gorski A. Is phage therapy acceptable in the immunocompromised host? Int J Infect Dis. 2008 Sep;12(5):466-71.
165. Slopek S, Weber-Dabrowska B, Dabrowski M, Kucharewicz-Krukowska A. Results of bacteriophage treatment of suppurative bacterial infections in the years 1981-1986. Arch Immunol Ther Exp (Warsz). 1987;35(5):569-83.
166. Slopek S, Durlakowa I, Weber-Dabrowska B, Kucharewicz-Krukowska A, Dabrowski M, Bisikiewicz R. Results of bacteriophage treatment of suppurative bacterial infections. I. General evaluation of the results. Arch Immunol Ther Exp (Warsz). 1983;31(3):267-91.
167. Slopek S, Durlakowa I, Weber-Dabrowska B, Kucharewicz-Krukowska A, Dabrowski M, Bisikiewicz R. Results of bacteriophage treatment of suppurative bacterial infections. II. Detailed evaluation of the results. Arch Immunol Ther Exp (Warsz). 1983;31(3):293-327.
168. Slopek S, Durlakowa I, Weber-Dabrowska B, Dabrowski M, Kucharewicz-Krukowska A. Results of bacteriophage treatment of suppurative bacterial infections. III. Detailed evaluation of the results obtained in further 150 cases. Arch Immunol Ther Exp (Warsz). 1984;32(3):317-35.
169. Slopek S, Kucharewicz-Krukowska A, Weber-Dabrowska B, Dabrowski M. Results of bacteriophage treatment of suppurative bacterial infections. IV. Evaluation of the results obtained in 370 cases. Arch Immunol Ther Exp (Warsz). 1985;33(2):219-40.
170. Slopek S, Kucharewicz-Krukowska A, Weber-Dabrowska B, Dabrowski M. Results of bacteriophage treatment of suppurative bacterial infections. V. Evaluation of the results obtained in children. Arch Immunol Ther Exp (Warsz). 1985;33(2):241-59.
171. Slopek S, Kucharewicz-Krukowska A, Weber-Dabrowska B, Dabrowski M. Results of bacteriophage treatment of suppurative bacterial infections. VI. Analysis of treatment of suppurative staphylococcal infections. Arch Immunol Ther Exp (Warsz). 1985;33(2):261-73.
172. Babalova EG, Katsitadze KT, Sakvarelidze LA, Imnaishvili N, Sharashidze TG, Badashvili VA, et al. [Preventive value of dried dysentery bacteriophage]. Zh Mikrobiol Epidemiol Immunobiol. 1968 Feb;45(2):143-5.
173. Sulakvelidze A, Alavidze Z, Morris JG, Jr. Bacteriophage therapy. Antimicrob Agents Chemother. 2001 Mar;45(3):649-59.
174. Larkum NW. Bacteriophage From a Public Health Standpoint. Am J Public Health Nations Health. 1929 Jan;19(1):31-6.
175. King WE, Boyd DA, Conlin JH. The cause of local reactions following the administration of Staphylococcus bacteriophage. Am J Clin Pathol. 1934;4:336-45.
176. Bogovazova GG, Voroshilova NN, Bondarenko VM, Gorbatkova GA, Afanas'eva EV, Kazakova TB, et al. [Immunobiological properties and therapeutic effectiveness of preparations from Klebsiella bacteriophages]. Zh Mikrobiol Epidemiol Immunobiol. 1992 Mar(3):30-3.
177. Capparelli R, Ventimiglia I, Roperto S, Fenizia D, Iannelli D. Selection of an Escherichia coli O157:H7 bacteriophage for persistence in the circulatory system of mice infected experimentally. Clin Microbiol Infect. 2006 Mar;12(3):248-53.
178. Bruttin A, Brussow H. Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. Antimicrob Agents Chemother. 2005 Jul;49(7):2874-8.
179. Ochs HD, Nonoyama S, Farrington ML, Fischer SH, Aruffo A. The role of adhesion molecules in the regulation of antibody responses. Semin Hematol. 1993 Oct;30(4 Suppl 4):72-9; discussion 80-1.

180. Bearden CM, Agarwal A, Book BK, Vieira CA, Sidner RA, Ochs HD, et al. Rituximab inhibits the in vivo primary and secondary antibody response to a neoantigen, bacteriophage phiX174. Am J Transplant. 2005 Jan;5(1):50-7.
181. Ochs HD, Davis SD, Wedgwood RJ. Immunologic responses to bacteriophage phi-X 174 in immunodeficiency diseases. J Clin Invest. 1971 Dec;50(12):2559-68.
182. Wedgwood RJ, Ochs HD, Davis SD. The recognition and classification of immunodeficiency diseases with bacteriophage phiChi 174. Birth Defects Orig Artic Ser. 1975;11(1):331-8.
183. Fogelman I, Davey V, Ochs HD, Elashoff M, Feinberg MB, Mican J, et al. Evaluation of CD4+ T cell function In vivo in HIV-infected patients as measured by bacteriophage phiX174 immunization. J Infect Dis. 2000 Aug;182(2):435-41.
184. Intralytix Inc. Food additive petition for: Control of pathogens on meat by use of a mixture of bacteriophages. 2002.
185. Salmon GG, Jr., Symonds M. Staphage Lysate Therapy in Chronic Staphylococcal Infections. J Med Soc N J. 1963 May;60:188-93.
186. Sulakvelidze A, Kutter E. Phage therapy in animals and agribusiness. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: biology and applications. 1st ed. Boca Raton, USA: CRC Press; 2005. p. 335-80.
187. Ryan EM, Gorman SP, Donnelly RF, Gilmore BF. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. J Pharm Pharmacol. 2011 Oct;63(10):1253-64.
188. Levin BR, Bull JJ. Phage therapy revisited: the population biology of a bacterial infection and its treatment with bacteriophage and antibiotics. Amer Naturalist. 1996;147(6):881-98.
189. Barrow PA, Soothill JS. Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. Trends Microbiol. 1997 Jul;5(7):268-71.
190. Kutter E. Phage therapy: bacteriophage as natural self-limiting antibiotics. In: Pizzorno JE, Murray MT, editors. Textbook of natural medicine. 4th ed. Oxford, UK: Churchill Livingstone; 2001. p. 945-56.
191. Soothill JS. Treatment of experimental infections of mice with bacteriophages. J Med Microbiol. 1992 Oct;37(4):258-61.
192. Soothill JS. Bacteriophage prevents destruction of skin grafts by Pseudomonas aeruginosa. Burns. 1994 Jun;20(3):209-11.
193. Goode D, Allen VM, Barrow PA. Reduction of experimental Salmonella and Campylobacter contamination of chicken skin by application of lytic bacteriophages. Appl Environ Microbiol. 2003 Aug;69(8):5032-6.
194. Kumari S, Harjai K, Chhibber S. Topical treatment of Klebsiella pneumoniae B5055 induced burn wound infection in mice using natural products. J Infect Dev Ctries. 2010 Jun;4(6):367-77.
195. Carmody LA, Gill JJ, Summer EJ, Sajjan US, Gonzalez CF, Young RF, et al. Efficacy of bacteriophage therapy in a model of Burkholderia cenocepacia pulmonary infection. J Infect Dis. 2010 Jan 15;201(2):264-71.
196. Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, et al. Bacteriophages can treat and prevent Pseudomonas aeruginosa lung infections. J Infect Dis. 2010 Apr 1;201(7):1096-104.
197. Hawkins C, Harper D, Burch D, Anggard E, Soothill J. Topical treatment of Pseudomonas aeruginosa otitis of dogs with a bacteriophage mixture: a before/after clinical trial. Vet Microbiol. 2010 Dec 15;146(3-4):309-13.

198. Matsuzaki S, Yasuda M, Nishikawa H, Kuroda M, Ujihara T, Shuin T, et al. Experimental protection of mice against lethal Staphylococcus aureus infection by novel bacteriophage phi MR11. J Infect Dis. 2003 Feb 15;187(4):613-24.
199. Brüssow H. Phage therapy: the western perspective. In: McGrath S, van Sinderen D, editors. Bacteriophage: genetics and microbiology 1st ed. Norfolk, UK: Caister Academic Press; 2007. p. 159-92.
200. Gorski A, Borysowski J, Miedzybrodzki R, Weber-Dabrowska B. Bacteriophages in medicine. In: McGrath S, van Sinderen D, editors. Bacteriophage: genetics and microbiology. 1st ed. Norfolk, UK: Caister Academic Press; 2007. p. 159-92.
201. Gorski A, Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Lobocka M, Fortuna W, et al. Bacteriophage therapy for the treatment of infections. Curr Opin Investig Drugs. 2009 Aug;10(8):766-74.
202. Harper DR, Kutter E. Bacteriophage: therapeutic use. The encyclopedia of life sciences. 1st ed. Chichester, UK: John Wiley & Sons; 2009. p. 1-7.
203. Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Fortuna W, Letkiewicz S, Szufnarowski K, et al. Clinical aspects of phage therapy. Adv Virus Res. 2012;83:73-121.
204. Letkiewicz S, Miedzybrodzki R, Kłak M, Weber-Dabrowska B, Górski A. Pathogen eradication by phage therapy in patients with chronic bacterial prostatitis (poster n.º 374). 25th Anniversary European Association of Urology Congress; Barcelona: European Association of Urology Congress; 2010.
205. Gorski A, Targonska M, Borysowski J, Weber-Dabrowska B. The potential of phage therapy in bacterial infections of the eye. Ophthalmologica. 2009;223(3):162-5.
206. Fortuna W, Miedzybrodzki R, Weber-Dabrowska B, Gorski A. Bacteriophage therapy in children: facts and prospects. Med Sci Monit. 2008 Aug;14(8):RA126-32.
207. Weber-Dabrowska B, Mulczyk M, Gorski A. Bacteriophage therapy of bacterial infections: an update of our institute's experience. Arch Immunol Ther Exp (Warsz). 2000;48(6):547-51.
208. Kurzepa A, Dabrowska K, Skaradzinski G, Gorski A. Bacteriophage interactions with phagocytes and their potential significance in experimental therapy. Clin Exp Med. 2009 Jun;9(2):93-100.
209. Kurzepa-Skaradzinska A, Lusiak-Szelachowska M, Skaradzinski G, Jonczyk-Matysiak E, Weber-Dabrowska B, Zaczek M, et al. Influence of bacteriophage preparations on intracellular killing of bacteria by human phagocytes in vitro. Viral Immunol. 2013 Apr;26(2):150-62.
210. Chanishvili N, Sharp R. A literature review of the practical application of bacteriophage research. 1st ed. Tbilisi, Georgia: Eliava Institute; 2009.
211. Markoishvili K, Tsitlanadze G, Katsarava R, Morris JG, Jr., Sulakvelidze A. A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. Int J Dermatol. 2002 Jul;41(7):453-8.
212. Jikia D, Chkhaidze N, Imedashvili E, Mgaloblishvili I, Tsitlanadze G, Katsarava R, et al. The use of a novel biodegradable preparation capable of the sustained release of bacteriophages and ciprofloxacin, in the complex treatment of multidrug-resistant Staphylococcus aureus-infected local radiation injuries caused by exposure to Sr90. Clin Exp Dermatol. 2005 Jan;30(1):23-6.
213. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care. 2009 Jun;18(6):237-8, 40-3.
214. Verbeken G, De Vos D, Vaneechoutte M, Merabishvili M, Zizi M, Pirnay JP. European regulatory conundrum of phage therapy. Future Microbiol. 2007 Oct;2(5):485-91.

215. Vandersteegen K, Mattheus W, Ceyssens PJ, Bilocq F, De Vos D, Pirnay JP, et al. Microbiological and molecular assessment of bacteriophage ISP for the control of Staphylococcus aureus. PLoS One. 2011;6(9):e24418.
216. 59th World Medical Association General Assembly. Ethical principles for medical research involving human subjects. World Medical Association Declaration of Helsinki: Available from: http://www.wma.net/en/30publications/10policies/b3/17c.pdf.
217. Wood JM, Levandowski RA. The influenza vaccine licensing process. Vaccine. 2003 May 1;21(16):1786-8.
218. Thiel K. Old dogma, new tricks--21st Century phage therapy. Nat Biotechnol. 2004 Jan;22(1):31-6.
219. Verbeken G, Pirnay JP, De Vos D, Jennes S, Zizi M, Lavigne R, et al. Optimizing the European regulatory framework for sustainable bacteriophage therapy in human medicine. Arch Immunol Ther Exp (Warsz). 2012 Jun;60(3):161-72.
220. Skurnik M, Pajunen M, Kiljunen S. Biotechnological challenges of phage therapy. Biotechnol Lett. 2007 Jul;29(7):995-1003.
221. Lineaweaver W, Howard R, Soucy D, McMorris S, Freeman J, Crain C, et al. Topical antimicrobial toxicity. Arch Surg. 1985 Mar;120(3):267-70.
222. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. Bacteriophage. 2011 Mar;1(2):111-4.
223. Stratton CW. Dead bugs don't mutate: susceptibility issues in the emergence of bacterial resistance. Emerg Infect Dis. 2003 Jan;9(1):10-6.
224. Alisky J, Iczkowski K, Rapoport A, Troitsky N. Bacteriophages show promise as antimicrobial agents. J Infect. 1998 Jan;36(1):5-15.
225. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999 May 21;284(5418):1318-22.
226. Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Gorski A. Phage therapy of staphylococcal infections (including MRSA) may be less expensive than antibiotic treatment. Postepy Hig Med Dosw (Online). 2007;61:461-5.
227. Cars O, Hogberg LD, Murray M, Nordberg O, Sivaraman S, Lundborg CS, et al. Meeting the challenge of antibiotic resistance. BMJ. 2008;337:a1438.
228. ECDC/EMEA Joint Working Group. The bacterial challenge: time to react. Stockholm, Sweden: European Centre for Disease Prevention and Control; 2009.
229. Kuchment A. The forgotten cure: the past and future of phage therapy. 1st ed. New York, USA: Springer; 2011.

Section B

Chapter 1: Epidemiological survey of diabetic foot infections

Specific framework

The first steps in all BT protocols involve some combination of bacteriophage isolation and selection (1-3). In our particular case, we already possessed a relatively large bacteriophage library because we were working directly with a company (TechnoPhage, S.A.) that develops bacteriophage-based products. However, we needed to develop an epidemiological study to choose bacterial targets and assemble a bacterial library against which we could test the bacteriophages. Therefore, we conducted a transversal observational study at four clinical centers in Lisbon (2 outpatient clinics, 1 general surgery ward and 1 vascular surgery ward) for a 6-month period. Clinical data was collected using a structured questionnaire (Annex B1.0-1) and microbiological products (aspirates, biopsies, or swabs collected using the Levine method) for clinically infected foot ulcers of patients with DM, as advised by current clinical guidelines (4). All health care providers (HCP) were instructed about the proper methods for the collection of culture material, and a written protocol was provided (Annex B1.0-2). Microbiological analysis was done at the Microbiology Laboratory of the Faculty of Veterinary Medicine, University of Lisbon. Sample processing, isolation, quantification, and identification of aerobic and anaerobic bacteria as well as antimicrobial susceptibility testing of the aerobic isolates were performed using the standard methods (5, 6). As quantitative cultures were performed, clinically relevant tissue burden (CRTB; swab count of $>10^5$ CFU/cm^2, tissue count of $>10^5$ CFU/g or a needle aspiration sample of $>10^5$ CFU/mL) was used as a potential indicator of the microorganisms' relevance in clinically infected DFUs. All isolated strains were stored, and clinical and microbiological information was compiled in a centralized database and later analyzed (Figure B1.0-1).

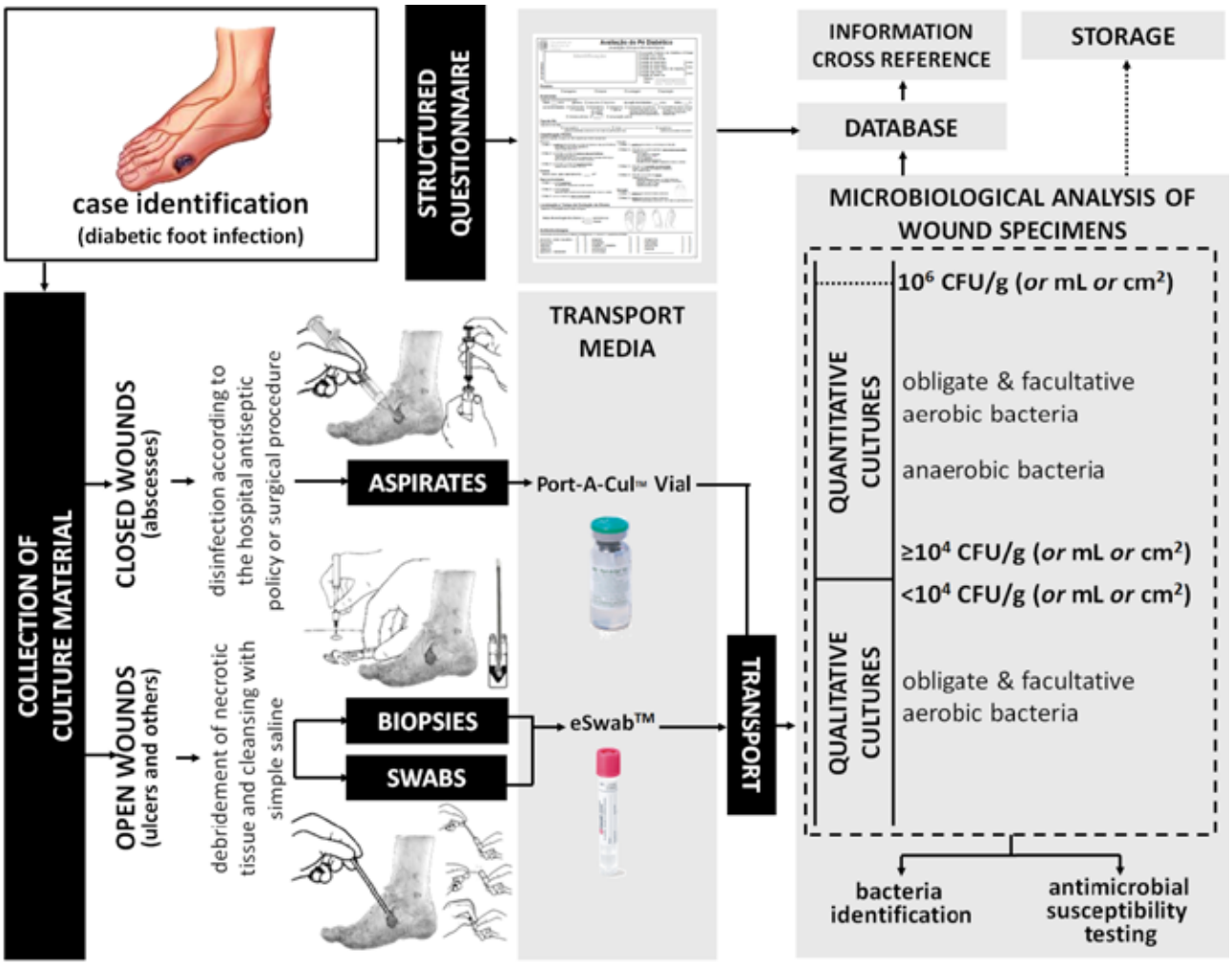


Figure B1.0-1 – Study outline of epidemiological survey of diabetic foot infections. All patients with diabetes mellitus and clinically infected foot ulcers presenting to any of four clinical centers in Lisbon were included in the study by their health care providers (HCP). A diabetic foot ulcer was defined as a full-thickness wound below the ankle in a diabetic patient, irrespective of duration (7). Infection was defined clinically by symptoms and signs of inflammation as described by the infection item on the PEDIS system (7). Medical histories, examination details, and investigation reports were recorded using a structured questionnaire. Specimens were collected as advised by current clinical guidelines (4). In the case of abscess with intact integument (and other closed lesions), the protocol suggested sampling by needle aspiration under strict aseptic technique. For ulcers and other open wounds, biopsy specimens were required, except in situations where the HCP considered that the invasive procedure could place the patient at risk (pain induction or risk of enlarging the ulcer). In either procedure, debridement of necrotic tissue and cleansing with simple saline before sampling was obligatory. In biopsies, shaving or punch techniques (8) were required. In swab sampling, HCPs were instructed in a standardized procedure (9), based on the Levine 1 cm^2 swab method, using a flocked swab. Transport of all samples in specific transport media to the laboratory was assured by an on-call express courier. Sample processing, isolation, quantification, and identification of aerobic and anaerobic bacteria as well as antimicrobial susceptibility testing of the aerobic isolates were performed using the standard methods (5, 6). All isolated strains were stored, and clinical and microbiological information was compiled in a centralized database and later analyzed.

References

1. Gill JJ, Hyman P. Phage choice, isolation, and preparation for phage therapy. Curr Pharm Biotechnol. 2010 Jan;11(1):2-14.
2. Goodridge LD. Designing phage therapeutics. Curr Pharm Biotechnol. 2010 Jan;11(1):15-27.
3. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011 Mar;1(2):66-85.
4. National Institute for Health and Clinical Excellence. NICE clinical guideline 119: Inpatient management of diabetic foot problems. London, UK: National Institute for Health and Clinical Excellence; 2011.
5. Weinstein MP. Diagnostic Technologies in Clinical Microbiology. In: Murray PR, Baron EJ, Jorgensen B, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology. 8th ed. Washington DC, USA: ASM Press; 2003. p. 183-1213.
6. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests - M02-A10 (Includes M100-S20). Wayne, USA: Clinical and Laboratory Standards Institute; 2010.
7. Schaper NC. Diabetic foot ulcer classification system for research purposes: a progress report on criteria for including patients in research studies. Diabetes Metab Res Rev. 2004 May-Jun;20 Suppl 1:S90-5.
8. Alavi A, Niakosari F, Sibbald RG. When and how to perform a biopsy on a chronic wound. Adv Skin Wound Care. 2010 Mar;23(3):132-40; quiz 41-2.
9. Bonham PA. Swab cultures for diagnosing wound infections: a literature review and clinical guideline. J Wound Ostomy Continence Nurs. 2009 Jul-Aug;36(4):389-95.

Clinical and bacteriological survey of diabetic foot infections in Lisbon.

Mendes JJ, Marques-Costa A, Vilela C, Neves J, Candeias N, Cavaco-Silva P, Melo-Cristino J.
Diabetes Res Clin Pract. 2012 Jan;95(1):153-61. doi: 10.1016/j.diabres.2011.10.001.
Epub 2011 Oct 21.
PMID: 22019426

Abstract

Aims: An epidemiological survey of diabetic foot infections (DFIs) in Lisbon, stratifying the bacterial profile based on patient demographical data, diabetic foot characteristics (PEDIS classification), ulcer duration and antibiotic therapy.

Methods: A transversal observational multicenter study, with clinical data collection using a structured questionnaire and microbiological products (aspirates, biopsies or swabs collected using the Levine method) of clinically infected foot ulcers of patients with diabetes mellitus (DM).

Results: Forty-nine hospitalized and ambulatory patients were enrolled in this study, and 147 microbial isolates were cultured. *Staphylococcus* was the main genus identified, and methicillin-resistant *S. aureus* (MRSA) was present in 24.5% of total cases. In the clinical samples collected from patients undergoing antibiotic therapy, 93% of the antibiotic regimens were considered inadequate based on the antibiotic susceptibility test results. The average duration of an ulcer with any isolated multi-drug resistant (MDR) organism was 29 days, and previous treatment with fluoroquinolones was statistically associated with multi-drug resistance.

Conclusions: S. aureus was the most common cause of DFIs in our area. Prevalence and precocity of MDR organisms, namely MRSA, were high and were probably related to previous indiscriminate antibiotic use. Clinicians should avoid fluoroquinolones and more frequently consider the use of empirical anti-MRSA therapy.

Keywords: Epidemiology, Diabetic foot, Infection, Microbiology, Portugal

Introduction

Diabetes mellitus (DM) is a serious health problem that is rapidly expanding worldwide (1). One of the more frequent diabetic complications is diabetic foot, which results from a complex interaction between a number of risk factors. Neuropathy (with alterations in motor, sensitive and autonomic functions) has a central role, causing ulcerations because of trauma or excessive pressure on deformed feet that lack protective sensitivity (2). Once the protective layer of skin is broken, the deep tissues are exposed to bacterial colonization. Infections are facilitated by immunological deficits (especially in neutrophils), which are related to DM, and they rapidly progress to the deep tissues. Patients with DM frequently require minor or major amputations of the lower limbs (15 to 27%), and in more than 50% of cases, infection is the preponderant factor (2).

S. aureus is the most prevalent isolate in diabetic foot ulcers (DFUs), together with other aerobes (including *Staphylococcus epidermidis*, *Streptococcus* spp., *P. aeruginosa*, *Enterococcus* spp. and coliform bacteria) and anaerobes (3, 4). Because of the polymicrobial nature of diabetic foot infections (DFIs), Karchmer et al. (5) questioned the need for precisely defining the causative microorganism and suggested a treatment strategy based only on the knowledge of the general epidemiology. More recently, an increase in the incidence of multi-drug resistant (MDR) organisms, namely methicillin-resistant *S. aureus* (MRSA) and extended-spectrum β-lactamase (ESBL)-producing gram-negative bacteria, is threatening the outcome of anti-infectious therapy in the community and in hospitalized patients (4). Therefore, the current guidelines (6) and expert opinion (7) advise providers to obtain specimens for culture before initiating empiric antibiotic therapy to help with the selection of a definitive therapy.

Although Portugal has one of the highest prevalences of DM, lower extremity amputations (8) and MRSA skin and soft tissue infections (9) in Europe, there is virtually no data on the prevalence and characterization of DFIs. Therefore, we performed an epidemiological survey of DFIs in Lisbon, stratifying the bacterial profiles based on patient demographical data, characteristics of diabetic foot (PEDIS classification), ulcer duration and current and recent (≤3 months prior) antibiotic therapy.

Subjects, materials and methods

This transversal observational study was conducted at 4 clinical centers (2 outpatient clinics, 1 general surgery ward and 1 vascular surgery ward) in Lisbon from January 2010 to June 2010. A structured questionnaire was developed to record medical histories, examination details and investigation reports by health care providers (HCPs). Specimens were collected from patients with DM and clinically infected foot ulcers, as advised by current clinical guidelines (6). A DFU was defined as a full-thickness wound below the ankle in a diabetic patient, irrespective of duration (10). Infection was defined clinically by symptoms and signs of inflammation, as described by the infection item on the PEDIS system (10). Specimens were obtained from patients before the first dose of antibiotics or while under antibiotic therapy with progression of infection signs and clinical deterioration of the ulcer.

This study was approved by the Faculty of Medicine of the University of Lisbon Research Ethics Committee and the Portuguese Data Protection Authority, and written informed consent was obtained for every patient.

Clinical characterization

For clinical characterization, 9 study factors were recorded for each patient: age, gender, DM duration (from diagnosis), last HbA1c value (accepted if collected in the last 3 months), hypertension and dyslipidemia (as defined according to the American Diabetes Association (ADA) guidelines for the diabetic population

(11)), active tobacco abuse (defined as ≥20 packs in the previous year), presence of ischemic heart disease (defined as previous history of myocardial infarction, coronary artery bypass graft or percutaneous transluminal coronary angioplasty) and chronic renal failure (defined as calculated glomerular filtration rate <30 mL min^{-1} 1.73 m^{-2}, permanent renal replacement therapy or previous transplant).

Diabetic foot characterizations

For characterization of diabetic foot, we used the International Working Group of the Diabetic Foot PEDIS system (10), which classified all foot ulcers in subcategories of five main categories (perfusion, extent/size, depth/tissue loss, infection and sensation), according to strict criteria. For the definition of osteomyelitis, a minimum of a positive probe-to-bone test (12) was accepted, but clinicians were encouraged to substantiate their diagnosis with the appropriate imaging studies. The number of previous ulcers and previous minor (toe or part of the foot) or major (above the ankle) amputations was also recorded.

Antibiotic therapy

HCPs were asked to register all current and recent (over the previous 3 months) antibiotic therapies.

Collection of samples

All HCPs were instructed on the proper methods for the collection of culture material, and a written protocol was provided. In the case of abscess with intact integument (and other closed lesions), the protocol suggested sampling by needle aspiration under strict aseptic technique. For ulcers and other open wounds, biopsy specimens were required, except in situations where the HCP considered that the invasive procedure could place the patient at risk (pain induction or risk of enlarging the ulcer). In only these situations, superficial swab samples were accepted, in strict accordance with the National Institute for Health and Clinical Excellence diabetic foot guideline (6). For either of the procedures, debridement of necrotic tissue and cleansing with simple saline before sampling was obligatory. For biopsies, shaving or punch techniques, as previously described (13), were required. For swab sampling, HCPs were instructed on a standardized procedure (14), based on the Levine 1 cm^2 swab method, using a flocked swab (ESwab Collection System, Copan).

Transport

Aspirates were transported in buffered isotonic agar with reduction agent media (Port-A-Cul Vial, BD BBL), and biopsies and swabs were transported in modified liquid Amies medium (ESwab Preservation System, Copan). Transport to the laboratory (Microbiology Laboratory, Faculty of Veterinary Medicine, Technical University of Lisbon) within 2 hours of collection was assured by an on-call express courier.

Processing and microbiological analysis of wound specimens

Standard methods for sample processing and isolation and identification of aerobic and anaerobic bacteria were used (15). Biopsy samples were weighed to the nearest milligram in sterile Petri dishes and homogenized in PBS using a pearl jar. A 100-μL volume of the homogenate was used for serial dilutions in PBS. For aspirate samples, a 100-μL volume of the recovered fluid was directly used for serial dilutions in PBS. Swab samples were vortexed with the swab inside for 5 seconds, and then a 100-μL volume of the suspension was used for serial dilutions in PBS. Quantification was performed using the 10-fold serial dilution method (15), and 100 μL of each dilution was inoculated onto MacConkey agar (Merck)/Columbia ANC agar with 5% sheep blood (BioMérieux) and, in duplicate, in Schaedler agar with 5% sheep blood (BioMérieux). The first two plates were incubated under aerobic

conditions at 35°C for 24 to 48 hours, and the two Schaedler plates were incubated under anaerobic conditions (Anaerocult A, Merck) for 48 to 96 hours. Additionally, samples were inoculated in Brain Heart Infusion Broth (Difco, BHIB) to allow recovery of fastidious or low-concentration organisms. Isolates were identified by standard methods (15). In some instances, unusual strains were identified using partial 16S rRNA gene sequencing (16). Antimicrobial susceptibility testing of the aerobic isolates was performed using the standard disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (17). Quantitative results were expressed in CFU/mL for needle aspiration samples, CFU/g for biopsy samples and CFU/cm^2 for swab samples. Consistent with the study by Bill et al. (18) and the results of a recent systematic review (19), a swab count of >10^5 CFU/cm^2 was considered equivalent to a tissue count of >10^5 CFU/g or a needle aspiration sample of >10^5 CFU/mL; all of these values are considered to represent a clinically relevant tissue burden (CRTB).

Multidrug resistance profiles

Methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE) and other coagulase-negative *Staphylococcus* spp. (MRCN) were defined as strains phenotypically resistant to cefoxitin (by the disc diffusion method). Vancomycin-resistant *Enterococcus* spp. (VRE) were defined as strains that were phenotypically resistant to vancomycin. (ESBL)-producing gram-negative strains were phenotypically confirmed using the cephalosporin/clavulanate combination disk test (20). Multi-drug resistant (MDR) *P. aeruginosa* and *Acinetobacter baumannii* strains were defined as those resistant to at least three of six antibiotics, including amikacin, gentamicin, ciprofloxacin, piperacillin, ceftazidime and imipenem. Pan-drug resistant (PDR) *P. aeruginosa* and *A. baumannii/calcoaceticus* strains were defined as those sensitive only to colistin (21). All of these strains (MRSA, MRCN, VRE, [ESBL]-producing gram-negative bacteria, and MDR and PDR *P. aeruginosa* and *A. baumannii/calcoaceticus*) were considered to be MDR organisms.

Statistical analysis

Qualitative variables were expressed as percentages, and quantitative variables are expressed as means ± SD (standard deviation). Significance of the study variables was tested using Student's *t*-test, the Chi-square test or Fisher's exact test, where appropriate. A two-tailed *p* value of <0.05 was considered to be statistically significant. Additionally, the ulcer duration (in days) was stratified by microbial isolate and visually summarized in a box plot, with the boxes representing the lower and upper quartiles, the vertical line the median, the bars the minimum and maximum data points, and the solid diamond symbol the mean.

Results

A total of 49 patients (mean age of 62.7 ± 12.7 years and a male-to-female ratio of 6.8) were admitted during the study period. Their clinical and diabetic foot characteristics, stratified in accordance with the sample collection method, are shown in Table B1.1-1. Among these patients, the mean duration of DM was 23.0 ± 12.8 years, 26.5% had HbA1c levels <58 mmol/mol (<7.5%), >90% had hypertension and/or dyslipidemia, and 30.6% and 10.2% had ischemic heart disease and chronic renal failure, respectively. Two-thirds of the patients had undergone recent antibiotic therapy, and one-third was currently undergoing antibiotic therapy. The majority of the samples came from outpatients (65.3%), and swabbing was the most commonly used method (63.3%) for sample collection. However, 92.8% of hospitalized patients and all clinically suspected osteomyelitis patients had samples collected by an invasive technique. There were statistically significant differences in the isolation rates of

microorganisms from deep tissue samples and superficial swabs, with fewer aerobes per sample, in particular gram-positive bacteria (2.3 ± 1.0 vs. 1.3 ± 1.2), isolated from swabs, but there was no difference in the isolation rate of anaerobes or MDR organisms.

Table B1.1-1 – Clinical and microbiological characteristics of DFIs stratified by the sample collection method

	total (n = 49)	swab samples (n = 31)	deep tissue samples[a] (n = 18)
Hospitalization (%)	34.7%	12.9%	72.2%
Demographical data			
Age (years)	62.7 ± 12.7	60.2 ± 13.5	67.0 ± 10.1
Male gender (%)	83.7%	87.1%	77.8%
Diabetes mellitus			
Control of diabetes (HbA_{1c} <7%)	20.4%	16.1%	17.8%
Duration (years)	23.0 ± 12.8	22.5 ± 12.8	23.7 ± 13.1
Co-morbidities			
Hypertension (%)	93.9%	96.8%	88.9%
Dyslipidemia (%)	95.9%	93.4%	100%
Active tobacco abuse (%)	38.7%	32.2%	50.0%
Organ lesions			
Ischemic heart disease (%)	30.6%	35.5%	22.2%
Chronic renal failure (%)	10.2%	12.9%	5.6%
Diabetic foot characterization			
Number of previous ulcers	1.6 ± 1.5	1.9 ± 1.6	1.2 ± 1.2
Previous amputation (%)	46.9%	51.6%	38.9%
major	10.2%	9.7%	11.1%
minor	38.8%	45.2%	27.8%
Duration of present ulcer (days)	30.6 ± 31.9	33.4 ± 25.9	25.7 ± 40.5
Neuroischemic (%)	53.1%	54.8%	50.0%
Osteomyelitis (%)	30.6%	0.0%	83.3%
PEDIS			
Perfusion 1 (%)	44.9%	43.9%	46.6%
2 (%)	40.8%	40.7%	41.0%
3 (%)	14.3%	19.3%	12.4%
Extent (cm^2)	13.3 ± 56.9	1.2 ± 0.6	34.3 ± 91.7
Depth 1 (%)	18.4%	29.0%	0.1%
2 (%)	51.0%	71.0%	16.6%
3 (%)	30.6%	0.0%	83.3%
Infection 2 (%)	61.2%	87.1%	16.6%
3 (%)	36.7%	12.9%	77.7%
4 (%)	2.0%	0.0%	5.4%
Sensation 2 (%)	100%	100%	100%
Antibiotic therapy			
Previous (%)	65.3%	67.7%	61.2%
Current (%)	30.6%	23.0%	43.7%
Isolates			
Monomicrobial (%)	16.3%	12.9%	22.1%
Total number (per sample)	3.0 ± 1.4	3.2 ± 1.3	2.7 ± 1.4
Aerobes	2.5 ± 1.1	2.7 ± 0.9	2.3 ± 1.3
Gram-positive	2.0 ± 1.0	2.3 ± 1.0	1.6 ± 1.1
Gram-negative	0.6 ± 0.6	0.5 ± 0.5	0.7 ± 0.7
Anaerobes	0.4 ± 0.6	0.4 ± 0.6	0.3 ± 0.6
MDR organisms	0.6 ± 0.9	0.5 ± 0.8	0.9 ± 1.0

[a] biopsies (n = 14) and aspirates (n = 4) MDR – multi-drug resistant

Out of the 49 patients enrolled in this study, 147 microbial isolates (comprising 43 species) were cultured, which represents an average of 3.0 ± 1.4 organisms per sample. Systematic results are presented in Table B1.1-2. Aerobes were present in 98.0% of cases, with gram-positive bacteria comprising 66.0% of the total number of isolates. *Staphylococcus* was the main genus identified, with *S. aureus* present in 51% of the samples and in 94.1% of the cases with a CRTB. Coagulase-negative *Staphylococcus* spp. were the second most frequently encountered aerobic gram-positive isolates, with *Staphylococcus epidermidis* and *Staphylococcus lugdunesis* commonly associated with a CRTB. *Corynebacterium* spp. and other uncommon gram-positive bacteria were also identified but not in clinically significant quantities. *Streptococcus* spp. were infrequently (4.1%) isolated. Gram-negative aerobes comprised 19.0% of the isolated organisms, while *P. aeruginosa*, the single most predominant species, was isolated in only 12.2% of cases. *Proteus* spp. were the next most frequently recovered gram-negative bacteria, although largely (75.0%) in non-CRTB cases. *A. baumannii/ calcoaceticus* were identified in 8.2% of the cases and were the non-PDR species found exclusively in the non-CRTB cases. Anaerobes were found in 30.6% of patients, with *Peptostreptococcus* spp. accounting for 55.0% of all anaerobic isolates, followed by the *Bacteroides fragilis* group, which accounted for 25% of these isolates, but this last group was more frequently identified in non-CRTB. *Candida* spp. were infrequently encountered, representing only 1.4% of the total isolates.

MDR organisms were present in 38.8% of cases, while MRSA was found in 24.5% of patients, thereby making it the predominantly isolated pathogen. MRSE and other methicillin-resistant coagulase-negative *Staphylococci* were also identified but accounted for only 4.8% of the isolates. Gram-negative MDR organisms were identified in a total of 18.9% of the patients. Of the isolated *Acinetobacter baumanni* and *P. aeruginosa* strains, 38.5% were PDR, and the remainder were MDR.

Although a longitudinal study using sequential microbiological samples was not performed, visually representing the relationship between the microbial isolates and ulcer duration in a box plot graph (Figure B1.1-1) revealed a pattern: gram-positive bacteria appeared in ulcers of short duration, while anaerobes associated with either gram-positive or -negative organisms appeared in ulcers of longer duration. This finding was independent of previous or current antimicrobial therapy. The average duration of an ulcer with any isolated MDR organism was 29 days.

Table B1.1-2 – Distribution of the DFI isolates

	n		%		% (/patients)		CRTB	
Aerobes	**125**		**85.0%**		**98.0%**		**63.2%**	
Gram-positive	**97**		**66.0%**		**95.9%**		**64.9%**	
Staphylococcus spp.	54		36.7%		79.6%		66.7%	
S. aureus (MRSA)	32	(17)	21.8%	(11.6%)	51.0%	(24.5%)	93.8%	(94.1%)
Staphylococcus epidermidis (MRSE)	7	(3)	4.8%	(2.0%)	14.3%	(4.1%)	42.9%	(66.7%)
other coagulase-negative *Staphylococcus* spp. (MRCN) [a]	15	(3)	10.2%	(2.0%)	20.4%	(4.1%)	20.0%	(33.3%)
Streptococcus spp. [b]	6		4.1%		12.2%		100%	
Enterococcus spp. [c] (VRE)	13	(1)	8.8%	(0.7%)	20.4%	(2.0%)	76.9%	(100%)
Corynebacterium spp. [d]	12		8.2%		28.6%		50.0%	
other Gram-positives [e]	12		8.2%		22.4%		41.7%	
Gram-negative	**28**		**19.0%**		**51.0%**		**57.1%**	
Enterobacteriaceae	16		10.9%		16.3%		56.3%	
Escherichia coli	1		0.7%		2.0%		100%	
Klebsiella spp. (ESBL)	2	(1)	1.4%	(0.7%)	4.1%	(2.0%)	100%	(100%)
Proteus spp. [f]	8		5.4%		16.3%		25.0%	
other *Enterobacteriaceae* [g]	5		3.4%		4.1%		80.0%	
nonfermenting negative bacilli	12		8.2%		20.4%		58.3%	
MDR *P. aeruginosa* (PDR-PA)	7	(2)	4.8%	(1.4%)	12.2%	(4.1%)	71.4%	(100%)
MDR *A. baumannii/calcoaceticus* (PDR-AB)	5	(3)	3.4%	(2.0%)	8.2%	(6.1%)	40.0%	(66.7%)
Anaerobes	**20**		**13.6%**		**30.6%**		**75.0%**	
Peptostreptococcus spp.	11		7.5%		22.4%		100%	
Bacteroides fragilis group	5		3.4%		4.1%		20.0%	
Other anaerobes [h]	4		2.7%		4.1%		75.0%	
Yeasts [i]	**2**		**1.4%**		**4.1%**		**–**	

in brackets are the multi-drug resistant (MDR) organisms of each species; CRTB – clinically relevant tissue burden; MRSA – methicillin-resistant *S. aureus*; MRSE – methicillin-resistant *Staphylococcus epidermidis*; MRCN – methicillin-resistant coagulase-negative *Staphylococcus* spp. other than *Staphylococcus epidermidis*; VRE – vancomycin-resistant *Enterococci*; ESBL – extended-spectrum beta-lactamases producing *Enterobacteriaceae*; MDR – multi-drug resistant; PDR-PA / PDR-AB – pan-drug-resistant *A. baumannii* / pandrug-resistant *P. aeruginosa*

[a] *Staphylococcus lugdunensis* (n = 2) and other coagulase-negative *Staphylococcus* spp. (n = 13); [b] *Streptococcus agalactiae* (n = 3), *Streptococcus mitis* group (n = 1) and *Streptococcus dysgalactiae* (n = 2); [c] *Enterococcus faecalis* (n = 9) and *Enterococcus faecium* (n = 1); [d] *Corynebacterium amycolatum/striatum* (n = 9) and other *Corynebacterium* spp. (n = 3); [e] *Dermabacter hominis* (n = 1), *Leuconostoc* spp. (n = 1), *Arcanobacterium* spp. (n = 2), *Arthrobacter* spp. (n = 1), *Kocuria varians/ rosea* (n = 2), *Cellulomonas* spp. / *Micrococcus* spp. (n=1) and *Brevibacterium* spp. (n=4); [f] *Proteus mirabilis* (n=4) and *Proteus vulgaris* (n=4); [g] *Enterobacter* spp. (n = 1), *Serratia marcescens* (n = 2) and *Morganella morganii* (n = 2); [h] *Fusobacterium* spp. (n = 1), *Prevotella* spp. (n = 1), *Eggerthella* spp. (n = 1) and *Veinonella* spp. (n = 1); [i] *Candida albicans* (n = 1) and *Candida parapsilosis* (n = 1)

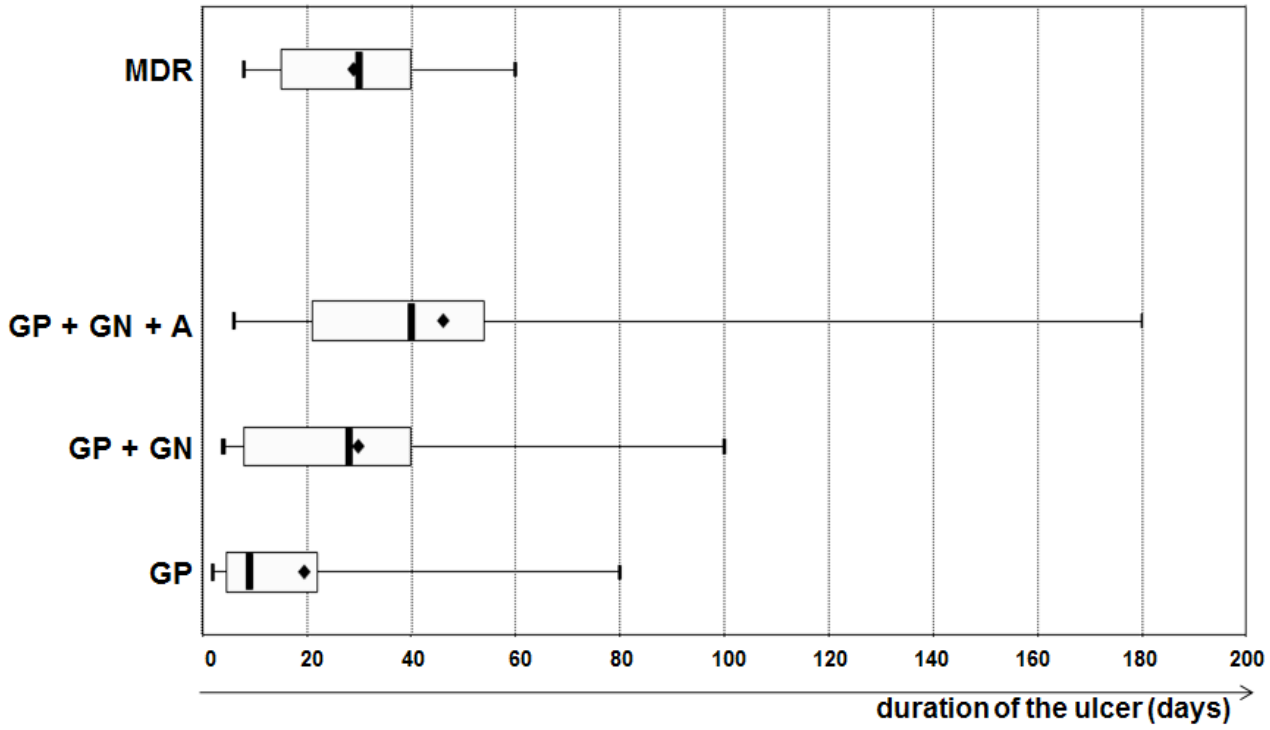


Figure B1.1-1 – A box plot representing the ulcer duration data (in days), stratified by the microbial isolate (the boxes represent the lower and upper quartiles, the vertical line the median, the bars the minimum and maximum data points, and the solid diamond symbol the mean).
MDROs – multi-drug resistant organisms, GP – gram-positive aerobes, GN – gram-negative aerobes, A – anaerobes

In the clinical samples collected from patients undergoing antibiotic therapy (Table B1.1-3), which corresponded mainly to hospitalized patients with osteomyelitis, 93% of the antibiotic regimens were considered inadequate based on the antibiotic susceptibility test results. Quantitative and qualitative differences were found in these samples, with fewer microorganisms identified (2.1 ± 0.9 vs. 3.4 ± 1.3); in particular, fewer gram-positive (86.7 vs. 100%) and anaerobic (6.7% vs. 41.2%) bacteria were identified; however, there was a higher prevalence of MDR organisms (66.7% vs. 26.5%). Although all the clinical variables were examined, multi-drug resistance was only statistically associated with current antibiotic treatment (with any class of antibiotics) and with previous fluoroquinolone treatment (Table B1.1-4).

Table B1.1-3 – Distribution of the DFI isolates in relation to current antibiotic therapy

	total (n = 49)	not under antibiotic therapy (n = 34)	under antibiotic therapy (n = 15)	*p* [a]
Hospitalization (%)	34.7%	17.6%	73.3%	<0.01
Isolates				
Total number (per sample)	3.0 ± 1.4	3.4 ± 1.3	2.1 ± 0.9	<0.01
Aerobes				
Number present per sample	2.5 ± 1.1	2.9 ± 1.0	1.9 ± 1.0	<0.01
Samples with ≥1 (%)	98.0%	100%	93.3%	NS
Gram-positive				
Number present per sample	2.0 ± 1.0	2.3 ± 1.0	1.5 ± 1.1	0.02
Samples with ≥1 (%)	95.9%	100%	86.7%	0.03
Gram-negative				
Number present per sample	0.6 ± 0.6	0.6 ± 0.6	0.4 ± 0.6	NS
Samples with ≥1 (%)	51.0%	58.8%	33.3%	NS
Anaerobes				
Number present per sample	0.4 ± 0.6	0.5 ± 0.6	0.1 ± 0.5	NS
Samples with ≥1 (%)	30.6%	41.2%	6.7%	0.01
MDR organisms				
Number present per sample	0.6 ± 0.9	0.4 ± 0.7	1.1 ± 1.0	<0.01
Samples with ≥1 (%)	38.8%	26.5%	66.7%	<0.01
Antibiotic therapy covers isolated pathogens	–	–	7.0% [b]	–

[a] not under antibiotic therapy vs. under antibiotic therapy

[b] of the total of patients current undergoing antibiotic therapy

Table B1.1-4 – Relationship between MDR organisms and recent (≤3 months) or current antibiotic therapy

	non-MDR (n = 30)	MDR [a] (n = 19)	*P* [b]
Previous antibiotic therapy	63.3%	73.7%	NS
Penicillins (including associations with b-lactamase inhibitors)	63.3%	79.0%	NS
Cephalosporins	13.3%	26.3%	NS
Carbapenems	10.0%	5.3%	NS
Aminoglycosides	0.0%	0.0%	NS
Sulphamides	13.3%	15.8%	NS
Fluoroquinolones	23.3%	63.2%	<0.01
Glycopeptides	6.7%	5.3%	NS
Oxazolidinones	0.0%	5.3%	NS
Others	3.3%	5.3%	NS
Current antibiotic therapy	16.7%	52.6%	<0.01
Penicillins (including associations with β-lactamase inhibitors)	6.7%	0.0%	NS
Cephalosporins	0.0%	0.0%	NS
Carbapenems	10.0%	15.8%	NS
Aminoglycosides	0.0%	5.3%	NS
Sulphamides	3.3%	0.0%	NS
Fluoroquinolones	10.0%	15.8%	NS
Glycopeptides	3.3%	5.3%	NS
Oxazolidinones	0.0%	5.3%	NS
Others	0.0%	5.3%	NS
Covers the isolated pathogens	40.0%	0.0%	0.03

[a] MRSA, MRSE, MRCN, VRE, ESBL-producing negatives, PDR *P. aeruginosa* and PDR *A. baumannii/ calcoaceticus*

[b] non-MDR vs. MDR

Discussion

DFIs are common, complex, and costly. They account for the largest number of proximate nontraumatic lower extremity amputations (2). This public health problem is particularly important in the underdiagnosed and undertreated diabetic Portuguese population (8). To our knowledge, this is the first published epidemiological study that reports the infectious microbiota and clinical characteristics of diabetic foot in patients located in Portugal. This study reflects the clinical profiles of inpatients and outpatients in the Lisbon area, but because the sample was relatively small, the study population was heterogeneous, and some controversial methodological issues were utilized (notably, the use of swabs and quantitative results), care must be taken when interpreting these results.

The baseline characteristics of the sample population are in line with those previously reported by European DFU studies (22), except for the high percentage of male patients and low percentage of patients with controlled DM (as evaluated by HbA1c). This can be partially explained by the hypothesis of a recent study (23) that reported that male gender and poor glycemic control are

independent risk factors for infection and non-healing DFUs. The high prevalence of co-morbidities is due to the low cut-offs used in the definitions.

Clinical guidelines (6) use infection severity and other clinical characteristics of DFUs as the basis for selecting an appropriate treatment approach, including antibiotic therapy. Our study used the PEDIS classification, and there were no statistical relationships between the diabetic foot characteristics, other than the duration of the ulcer and a clinical suspicion of osteomyelitis, and specific pathogens. We cannot be certain that the lack of significant associations was due only to the small sample size, however.

It is well documented in the literature (3, 4) that DFIs are polymicrobial in nature. In the present study, polymicrobial cultures were obtained from 83.7% of patients with a rate of isolation of 3.0 ± 1.4 bacteria per patient, independent of the sampling method, which is similar to the results seen in previous studies. In agreement with published western studies (3, 4), we isolated predominantly aerobic gram-positive cocci from acute infections, while a more complex flora, including gram-negative and anaerobic bacteria was obtained from chronic wounds.

We also found that *S. aureus*, either alone or as a component of a mixed infection, to be the most frequently isolated pathogen. Coagulase-negative *Staphylococcus* spp. were also frequently found, often with a methicillin-resistance phenotype. *Streptococcus* spp., which are well-recognized pathogens in DFIs, were infrequently isolated. This can be partially justified by the high prevalence of present and recent antibiotic therapy. *Enteroccoccus* spp., considered low-virulence commensal organisms, except in diabetic and other compromised patients, were identified in 20.4% of patients, which is in accordance with other studies (3, 4).

In strict accordance with other western studies (3, 4), but unlike studies from India and other Asian countries (24), we isolated relatively few aerobic gram-negative organisms. In our study, the high percentage of *P. aeruginosa* and low percentage of *Proteus* spp. isolates with a CRTB was consistent with the view that the first species can cause severe tissue damage in DM patients and should be regarded as significant in that population, while the latter are most commonly non-pathogenic (7).

Independent of the sampling method, anaerobes were isolated in one-third of the patients and almost always in mixed culture. This is in contrast to the findings of several other studies that failed to isolate anaerobes, possibly because of suboptimal study protocols (25). The anaerobes isolated from our study are consistent with other reported studies (26), in which *Peptostreptococcus* spp. were the predominant isolates. Although the exact role of anaerobic bacteria in DFIs is still under debate, our study is in line with the expert opinion (7) that suggests that anaerobes are more likely to be isolated from long-standing infections.

Other important factors to consider when interpreting the results of our study are that DFI is a clinical diagnosis and that both the quantitative and qualitative aspects of wound microbiology are critical determinants of an infection's course. All the patients enrolled in our study had clinically infected DFUs, and we based our conclusions on a qualitative microbiological analysis, considering the diversity of the microorganisms and the potential for microbial synergy, and on quantitative microbiological analysis, which provided a good indication of the microbial load. Assuming that the qualitative microbiology remains constant, the probability of wound infection increases with the microbial load, up to a critical level at which infection or a failure to heal is considered to be almost inevitable. In this paper, CRTB represented the quantitative aspect of wound microbiology and was used only as a potential indicator of the microorganisms' relevance in clinically infected DFUs.

One of the main limitations of our study is that the quantitative and qualitative microbial evaluations were predominantly performed using swab samples. While tissue biopsies and fluid

aspirates are considered the gold standard for diagnosing wound infections (25), these invasive tests are performed infrequently with small wounds and in many practice settings, such as outpatient clinics, due to concerns over enlarging the ulcer or inducing pain (14, 25, 27). In our study, we introduced a standardized procedure that was strictly consistent with the current clinical guidelines (6). Our method used quantitative aerobic and anaerobic swab cultures as an alternative method when the HCP believed an invasive procedure would place the patient at risk. While this decision was based on the microbiological experimental and clinical evidence supporting the hypothesis that the results form quantitative swabs are highly correlated with those from invasive procedures (sensitivities from 93.5% to 100% and specificities from 76.3% to 94.2% have been previously reported (14)), this hypothesis is not consensual in the scientific community. Some authors have reported consistency between swab and deep tissue biopsy sample cultures (28, 29), while others believe that superficial swab cultures of DFIs only complicate patient evaluation by sampling the superficial wound compartment, which may contain colonizing organisms rather than true pathogens. These divergent conclusions may be explained by different and non-standardized protocols. While we acknowledge that a standardized quantitative swab sampling protocol may be an imperfect and difficult-to-implement method in the clinical setting, it clearly has merits in the research field, at least in a setting with a high prevalence of the multi-drug resistance setting such as in our study; when properly interpreted, they can provide useful information (27).

We had a surprisingly high number of swab samples (mainly from outpatient clinics) from patients with small superficial ulcers. There were statistically significant differences between the superficial and deep samples, probably due to swab-associated and impossible-to-eliminate wound contamination by members of the endogenous microbiota (mainly gram-positive aerobes). This result may explain the high prevalence of *Corynebacterium* spp. and other low-virulence colonizers (e.g., *Dermabacter hominis* and *Leuconostoc* spp.), which were mainly cultured from swab samples.

In the present study, MDR organisms were cultured from 38.8% of the patients, the majority (24.5%) of which were MRSA. Most of the other international studies that have reported a similarly high percentage of MDR organisms were single-center, hospital-based studies (24). The high prevalence in such studies may be explained by the institution's use of broad spectrum antibiotics, resulting in a pathogen-selective survival advantage. In our multicenter study, we did not find any statistically significant differences between the inpatients and outpatients, and the mean duration of ulcers with isolated MDR organisms was short (29 days).

We also found a high percentage of patients (65.3%) who had received antibiotics in the previous three months and a statistical association between the presence of MDR organisms and previous fluoroquinolone therapy. This class of antibiotics has been widely used in Portugal for many years (30), and others have described (31) how they use correlates with the spread of MDR organisms, particularly MRSA. Therefore, our results suggest that multi-resistance in our area is widespread in diabetic patients with foot ulcers, and fluoroquinolone abuse (including inadequate dosing or suboptimal therapy duration) in the community could be a potential cause.

We also evaluated samples from DFI patients receiving antibiotic therapy, mainly hospitalized patients with osteomyelitis, who had signs of infection progression and clinical deterioration of their ulcers. Microbial isolation was significantly influenced by systemic antibiotic therapy, with fewer microorganisms (mostly anaerobic bacteria) identified but with a significantly greater prevalence of MDR organisms. This finding may be explained by selective pressure because the majority of these patients were under broad-spectrum antibiotic therapy, mostly with carbapenems. There are surprisingly few published clinical trials on antibiotic therapy for DFIs, and the available data do

not allow current guidelines to recommend any specific antibiotic regimen. In 2010, however, the Portuguese Directorate-General of Health (32) published a clinical guideline suggesting the use of isoxazolylpenicillins or clindamycin for superficial infections, aminopenicillins with a β-lactamase inhibitor or fluoroquinolones combined with clindamycin for deep infections, and carbapenems or ureidopenicillins with a β-lactamase inhibitor for more severe infections. The same guideline also considered the potential use of cotrimoxazole, vancomycin, linezolid or tigecycline if MRSA was suspected but did not mention any suspicion criteria. Although these guidelines are typically considered by HCPs, our study showed that the initial empirical antibiotic therapy covered the isolated pathogens of patients with clinically deteriorating ulcers in only 7.0% of the cases. Therapeutic failure was related to the presence of MDR organisms, namely MRSA.

In conclusion, our observational study provides a unique picture of the DFI pattern in our region. Both the prevalence and precocity of MDR organisms were alarmingly high and were probably related to indiscriminate antibiotic use. Fluoroquinolones, because of their pharmacological characteristics, safety and proven clinical effectiveness, are among the antimicrobial agents currently recommended by authoritative DFI guidelines. However, resistance has been directly linked to the use of these compounds, and the present study describes a statistical association that should encourage clinicians, and ultimately health authorities, to avoid their widespread use. By contrast, due to the high prevalence of MRSA in DFIs in our area, we suggest empirical anti-MRSA therapy followed by de-escalation to rationalize care and improve outcomes.

References

1. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract. 2010 Jan;87(1):4-14.
2. Richard JL, Sotto A, Lavigne JP. New insights in diabetic foot infection. World J Diabetes. 2011 Feb 15;2(2):24-32.
3. Citron DM, Goldstein EJ, Merriam CV, Lipsky BA, Abramson MA. Bacteriology of moderate-to-severe diabetic foot infections and in vitro activity of antimicrobial agents. J Clin Microbiol. 2007 Sep;45(9):2819-28.
4. Tascini C, Piaggesi A, Tagliaferri E, Iacopi E, Fondelli S, Tedeschi A, et al. Microbiology at first visit of moderate-to-severe diabetic foot infection with antimicrobial activity and a survey of quinolone monotherapy. Diabetes Res Clin Pract. 2011 Aug 6.
5. Karchmer AW, Gibbons GW. Foot infections in diabetes: evaluation and management. Curr Clin Top Infect Dis. 1994;14:1-22.
6. National Institute for Health and Clinical Excellence. NICE clinical guideline 119: Inpatient management of diabetic foot problems. London, UK: National Institute for Health and Clinical Excellence; 2011.
7. Joseph WS, Lipsky BA. Medical therapy of diabetic foot infections. J Am Podiatr Med Assoc. 2010 Sep-Oct;100(5):395-400.
8. Organization for economic co-operation and development. Health at a glance 2009: OECD indicators: Available from: www.oecd.org/dataoecd/32/34/43541373.pdf.
9. Moet GJ, Jones RN, Biedenbach DJ, Stilwell MG, Fritsche TR. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998-2004). Diagn Microbiol Infect Dis. 2007 Jan;57(1):7-13.
10. Schaper NC. Diabetic foot ulcer classification system for research purposes: a progress report on criteria for including patients in research studies. Diabetes Metab Res Rev. 2004 May-Jun;20 Suppl 1:S90-5.
11. Executive summary: standards of medical care in diabetes-2009. Diabetes Care. 2009 Jan;32 Suppl 1:S6-12.
12. Lavery LA, Armstrong DG, Peters EJ, Lipsky BA. Probe-to-bone test for diagnosing diabetic foot osteomyelitis: reliable or relic? Diabetes Care. 2007 Feb;30(2):270-4.
13. Alavi A, Niakosari F, Sibbald RG. When and how to perform a biopsy on a chronic wound. Adv Skin Wound Care. 2010 Mar;23(3):132-40; quiz 41-2.
14. Bonham PA. Swab cultures for diagnosing wound infections: a literature review and clinical guideline. J Wound Ostomy Continence Nurs. 2009 Jul-Aug;36(4):389-95.
15. Weinstein MP. Diagnostic Technologies in Clinical Microbiology. In: Murray PR, Baron EJ, Jorgensen B, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology. 8th ed. Washington DC, USA: ASM Press; 2003. p. 183-1213.
16. Woo PC, Leung AS, Leung KW, Yuen KY. Identification of slide coagulase positive, tube coagulase negative Staphylococcus aureus by 16S ribosomal RNA gene sequencing. Mol Pathol. 2001 Aug;54(4):244-7.
17. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests - M02-A10 (Includes M100-S20). Wayne, USA: Clinical and Laboratory Standards Institute; 2010.

18. Bill TJ, Ratliff CR, Donovan AM, Knox LK, Morgan RF, Rodeheaver GT. Quantitative swab culture versus tissue biopsy: a comparison in chronic wounds. Ostomy Wound Manage. 2001 Jan;47(1):34-7.
19. Nelson EA, O'Meara S, Craig D, Iglesias C, Golder S, Dalton J, et al. A series of systematic reviews to inform a decision analysis for sampling and treating infected diabetic foot ulcers. Health Technol Assess. 2006 Apr;10(12):iii-iv, ix-x, 1-221.
20. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev. 2005 Oct;18(4):657-86.
21. Falagas ME, Koletsi PK, Bliziotis IA. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) Acinetobacter baumannii and Pseudomonas aeruginosa. J Med Microbiol. 2006 Dec;55(Pt 12):1619-29.
22. Prompers L, Schaper N, Apelqvist J, Edmonds M, Jude E, Mauricio D, et al. Prediction of outcome in individuals with diabetic foot ulcers: focus on the differences between individuals with and without peripheral arterial disease. The EURODIALE Study. Diabetologia. 2008 May;51(5):747-55.
23. Shakil S, Khan AU. Infected foot ulcers in male and female diabetic patients: a clinico-bioinformative study. Ann Clin Microbiol Antimicrob. 2010;9:2.
24. Zubair M, Malik A, Ahmad J. Clinico-microbiological study and antimicrobial drug resistance profile of diabetic foot infections in North India. Foot (Edinb). 2011 Mar;21(1):6-14.
25. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001 Apr;14(2):244-69.
26. Ng LS, Kwang LL, Yeow SC, Tan TY. Anaerobic culture of diabetic foot infections: organisms and antimicrobial susceptibilities. Ann Acad Med Singapore. 2008 Nov;37(11):936-9.
27. Dow G. Bacterial swabs and the chronic wound: when, how, and what do they mean? Ostomy Wound Manage. 2003 May;49(5A Suppl):8-13.
28. Pellizzer G, Strazzabosco M, Presi S, Furlan F, Lora L, Benedetti P, et al. Deep tissue biopsy vs. superficial swab culture monitoring in the microbiological assessment of limb-threatening diabetic foot infection. Diabet Med. 2001 Oct;18(10):822-7.
29. Slater RA, Lazarovitch T, Boldur I, Ramot Y, Buchs A, Weiss M, et al. Swab cultures accurately identify bacterial pathogens in diabetic foot wounds not involving bone. Diabet Med. 2004 Jul;21(7):705-9.
30. Ferech M, Coenen S, Malhotra-Kumar S, Dvorakova K, Hendrickx E, Suetens C, et al. European Surveillance of Antimicrobial Consumption (ESAC): outpatient quinolone use in Europe. J Antimicrob Chemother. 2006 Aug;58(2):423-7.
31. Muller A, Mauny F, Talon D, Donnan PT, Harbarth S, Bertrand X. Effect of individual- and group-level antibiotic exposure on MRSA isolation: a multilevel analysis. J Antimicrob Chemother. 2006 Oct;58(4):878-81.
32. Direcção Geral da Saúde. [Diabetic foot. National program for prevention and control of diabetes]. Clinical Guideline nº:05/PNPCD. 2010.

Chapter 2: In vitro design of the bacteriophage cocktail

Specific framework

The development of an effective BT protocol is a toilsome, multi-step process (1). Once the target bacteria are defined, it is necessary to isolate and select the appropriate bacteria-specific lytic bacteriophages. These must then be morphologically and genetically characterized, mainly because of safety issues. Thereafter, as in any antibiotic development, in vitro studies precede in vivo studies and are employed to design a pharmacologically-informed dosage regimen. Several in vitro studies using multiple bacteriophages with diverse bacterial hosts intended for human applications have been published (2-4). The majority of these studies combine two or more bacteriophages (bacteriophage cocktails) in order to achieve effectiveness under a greater diversity of conditions and/or target more bacterial strains. Some also present associated in vivo efficacy studies (5). However, most report only the morphological and/or genetic characterization of the bacteriophages used, excluding pharmacological information of the diverse formulations. There are also relevant data available about preparations developed for non-clinical applications (6, 7). In our case, Technophage, S.A., had already isolated *S. aureus*, *P. aeruginosa* and *A. baumannii/calcoaceticus* specific-bacteriophages from environmental samples. Their morphological and genetic structures were investigated, and they were tested against clinical isolates (including 44 DFIs isolates from the epidemiological study) using the bacteriophage spot test procedure (8). Subsequently, their activity against planktonic cells and established biofilms was studied, and these data were used to design a dosage regimen for use in future studies (Figure B2.0-1).

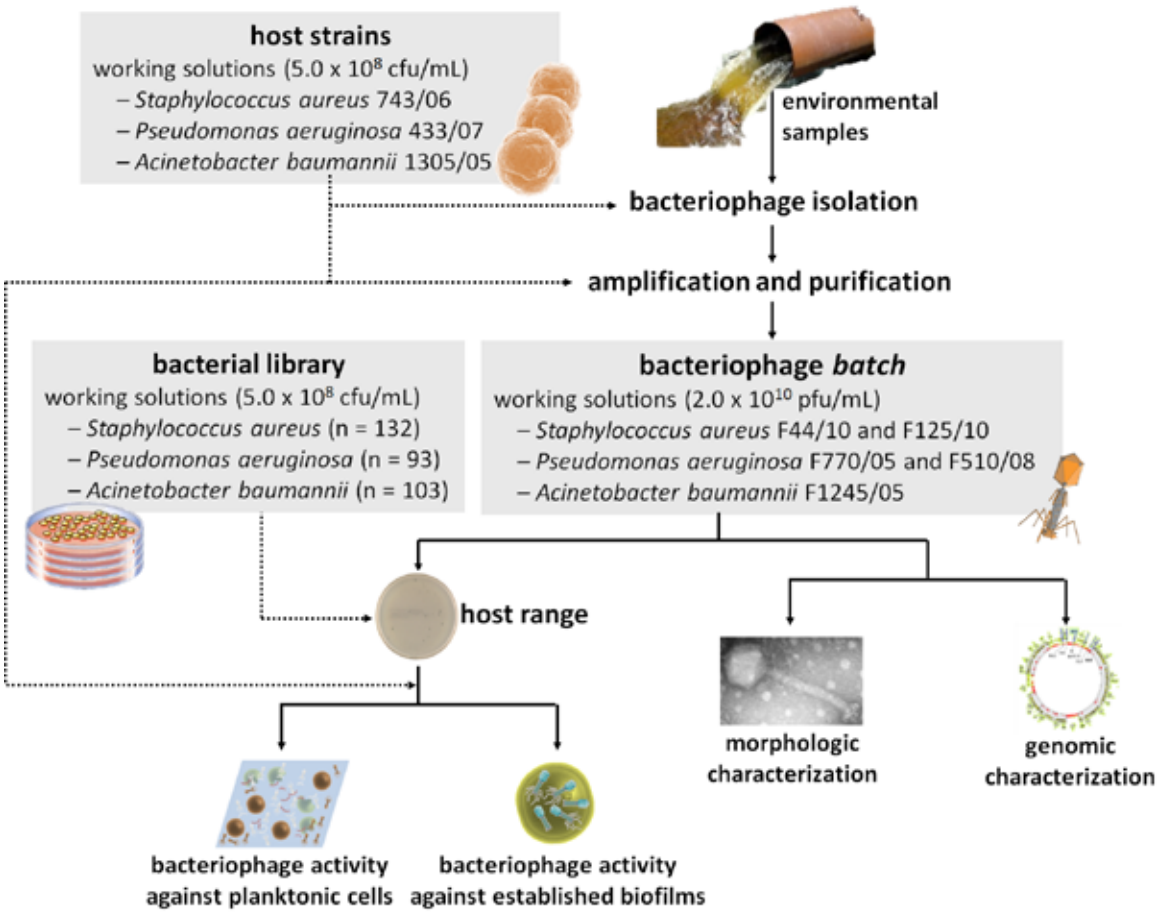


Figure B2.0-1 – In vitro design of a lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections. *S. aureus*, *P. aeruginosa*, and *A. baumannii*-specific lytic bacteriophages were isolated from sewage water from the Lisbon area by employing standard methods. To produce bacteriophage stocks in sufficient quantities for the experiments, a standard protocol of amplification, concentration by high-speed centrifugation, and purification on a cesium chloride gradient was used. Final concentrations were determined with double agar overlay plaque assays and further diluted to achieve a working solution titer of 10^{10} pfu/mL prior to the assays. For the morphological characterization, all the bacteriophages were analyzed by transmission electron microscopy at the Félix d'Hérelle Reference Center for Bacterial Viruses. For the genotypic characterization, the DNA of each of the 5 bacteriophages was isolated using a standard phenol/ chloroform extraction and precipitation protocol, sent for commercial sequencing, and analysed by extensive bioinformatics evaluation. All bacteriophages were subsequently tested against clinical isolates (including 44 DFI bacterial isolates from the epidemiological study) using the bacteriophage spot test procedure. In order to determine bacteriophage activity against planktonic cells in vitro, a kinetic time-kill assay was performed using a modified protocol. A quantification model based on the reduction of alamarBlue by metabolically active cells was then used to investigate the effect of the different bacteriophage combination on the preformed bacterial biofilms.

References

1. Abedon ST, Thomas-Abedon C. Phage therapy pharmacology. Curr Pharm Biotechnol. 2010 Jan;11(1):28-47.
2. Vandersteegen K, Mattheus W, Ceyssens PJ, Bilocq F, De Vos D, Pirnay JP, et al. Microbiological and molecular assessment of bacteriophage ISP for the control of Staphylococcus aureus. PLoS One. 2011;6(9):e24418.
3. Kesik-Szeloch A, Drulis-Kawa Z, Weber-Dabrowska B, Kassner J, Majkowska-Skrobek G, Augustyniak D, et al. Characterising the biology of novel lytic bacteriophages infecting multidrug resistant Klebsiella pneumoniae. Virol J. 2013;10:100.
4. Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Tediashvili M, Lashkhi N, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One. 2009;4(3):e4944.
5. Chan BK, Abedon ST. Phage therapy pharmacology phage cocktails. Adv Appl Microbiol. 2012;78:1-23.
6. Sillankorva S, Neubauer P, Azeredo J. Pseudomonas fluorescens biofilms subjected to phage phiIBB-PF7A. BMC Biotechnol. 2008;8:79.
7. Shen Y, Liu Y, Zhang Y, Cripe J, Conway W, Meng J, et al. Isolation and characterization of Listeria monocytogenes isolates from ready-to-eat foods in Florida. Appl Environ Microbiol. 2006 Jul;72(7):5073-6.
8. Armon R, Kott Y. A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. J Appl Bacteriol. 1993 Apr;74(4):490-6.

In vitro design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections

Mendes JJ, Leandro C, Mottola C, Barbosa R, Silva FA, Oliveira M, Vilela CL, Melo-Cristino J, Górski A, Pimentel M, São-José C, Cavaco-Silva P, Garcia M
J Med Microbiol. 2014 May 28. pii: jmm.0.071753-0. doi: 10.1099/jmm.0.071753-0.
[Epub ahead of print]
PMID: 24869663 [PubMed – as supplied by publisher]

Abstract
In patients with diabetes mellitus, foot infections pose a significant risk. These are complex infections commonly caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, all of which are potentially susceptible to bacteriophages. Here, we characterized five bacteriophages that we previously determined to have antimicrobial and wound-healing potential in chronic *S. aureus*, *P. aeruginosa*, and *A. baumannii* infections. Morphological and genetic features indicated that the bacteriophages were lytic members of *Myoviridae* or *Podoviridae* and did not harbor any known bacterial virulence genes. Combinations of the bacteriophages had broad host ranges for the different target bacterial species. The bacteriophages' activity against planktonic cells revealed effective, early killing at 4 hours, followed by bacterial regrowth to pretreatment levels by 24 hours. By using metabolic activity as a measure of cell viability within established biofilms, we found significant cell impairment following bacteriophage exposure. Repeated treatment every 4 hours caused a further decrease in cell activity. The greatest effects on both planktonic and biofilm cells occurred at a bacteriophage/bacterium input multiplicity (IM) of 10. These studies on both planktonic cells and established biofilms allowed us to better evaluate the effects of a high IM and a multiple-dose treatment protocol, and the findings support further clinical development of bacteriophage therapy.

Keywords: bacteriophage therapy, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, diabetic foot infections, in vitro, posology

Introduction

Diabetes mellitus affects an estimated 171 million patients worldwide (1) and has become a major world epidemic. Even with the best preventive care, 9% of patients will develop a diabetic foot infection (DFI), which brings the consequent risk of amputation (2). Qualitative and quantitative aspects of wound microbiology are critical determinants of the wound outcome. Gram-positive microorganisms are the first to colonize and acutely infect breaks in the skin, whereas chronic wounds develop a more complex polymicrobial microbiology, including aerobic Gram-negative rods (3). These microorganisms aggregate in communities encased within extracellular polymeric substances on the wound surface. Such an entity is defined as a biofilm, which shows increased resistance to immunological and antimicrobial attack (4). In current clinical practice, DFI treatment includes debridement and systemic antibiotics (3). The increased incidence of antibiotic-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* and pandrug-resistant, non-fermenting Gram-negative bacilli, threatens the efficacy of antimicrobial therapy (5). Thus, it is necessary to identify new therapeutic strategies for DFIs.

Bacteriophages are viruses that consist of a genome contained within a protein coat and that specifically infect bacteria. In contrast to filamentous bacteriophages, the multiplication of tailed bacteriophages and release of the newly formed virus particles always involves lysis of the host bacterial cell. However, among tailed bacteriophages some may not immediately follow this lytic pathway. The genome of these so called temperate bacteriophages may instead reside in the host cell (integrated in the bacterial chromosome or in a plasmid-like form in the cytoplasm) and be propagated for several bacterial generations without lysis. In contrast, strictly lytic phages do not have this option and usually undergo the lytic pathway once inside the bacterial host (6). Bacteriophage therapy (BT) is the use of lytic bacteriophages to reduce or eliminate pathogenic bacteria. BT has become a broadly relevant technology for veterinary, agricultural, and food microbiological applications; however, the treatment of human infections with BT attracts the greatest interest (7).

The use of bacteriophages as antibacterial agents for suppurative infections began shortly after the discovery of bacteriophages. Bruynoghe and Maisin first demonstrated BT, using bacteriophages to treat *S. aureus* skin infections (8). However, following the discovery and general application of antibiotics, interest in the therapeutic uses of bacteriophages waned. Recently, the increase in antibiotic-resistant bacterial strains has reinvigorated enthusiasm about these bacteria-specific viruses (9). This interest is particularly true in cases in which bacteriophages can be applied externally (topical application), as is the case for DFIs.

The development of an effective BT is a multi-step process consisting of (1) bacteriophage isolation and assessment for antibacterial activity against specific bacterial strains, (2) bacteriophage characterization and screening for undesirable traits, (3) in vitro posology and dosage regimen design, (4) preclinical animal efficacy and toxicology studies, and (5) regulated human clinical trials. Although the use of bacteriophages to treat DFIs is promising, difficulties in any of these steps can hinder widespread clinical application (10).

Recently, we demonstrated the antimicrobial activity and wound-healing capability of a topically delivered bacteriophage suspension against wounds chronically infected with chronic *S. aureus, Pseudomonas aeruginosa*, and *Acinetobacter baumannii* in two animal models of diabetes mellitus (11). In the current study, we present a characterization of the bacteriophages used in the previous study. We examined their spectrum of activity, genetic and morphological structures, and activity against planktonic cells and established biofilms. Collectively, the findings justify the posology and dosage regimen used in the animal studies.

Materials and methods

Bacterial strains

The *S. aureus* 743/06, *P. aeruginosa* 433/07, and *A. baumannii* 1305/05 host strains were isolated from human clinical samples that were collected and identified in hospitals in the Lisbon area. The three strains were previously characterized as biofilm producers (12). Bacterial clinical isolates used for bacteriophage host-range investigation included *S. aureus* (n = 132), *P. aeruginosa* (n = 93), and *A. baumannii* (n = 103) from wound specimens. Of these isolates, 44 were from DFIs. The epidemiology, clinical details, and specific microbiology of our collection of DFI isolates have been previously described (5). All isolates were stored in tryptone soy broth (TSB; Biokar Diagnostics, Pantin Cedex, France) with 15% glycerol (w/v) at -70°C until needed. For the experiments, single bacterial colonies were grown in TSB at 37°C. After a 24-hour incubation, the bacterial cells were suspended in saline and adjusted to McFarland's scale 0.5 (bioMérieux, Craponne, France), producing a final working suspension of approximately 5.0×10^8 cfu/ml.

Bacteriophages

Bacteriophage isolation, amplification, and purification

S. aureus F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 bacteriophages were isolated from environmental water samples from the Lisbon area. Standard methods for bacteriophage isolation (13) were employed for all five bacteriophages using the host strains described above. The obtained bacteriophage plaques were purified by repeated single plaque isolation to ensure that each contained only one type of bacteriophage.

To produce bacteriophage stocks in sufficient quantities for the experiments, a previously described protocol of amplification, concentration by high-speed centrifugation, and purification on a cesium chloride gradient (14) was used for all five bacteriophages. Briefly, a final lysate of each bacteriophage was centrifuged at 10,000 × g for 20 minutes at 4°C. The pellet was discarded, and the supernatant fraction was concentrated overnight at 8,000 rpm (rotor JA-14, Beckman Coulter, Fullerton, USA). The bacteriophage pellet was resuspended in SM buffer (5.8 g l^{-1} NaCl, 2 g l^{-1} $MgSO_4$ × 7 H_2O, 50 ml 1 M Tris, pH 7.5). This concentrated bacteriophage suspension was loaded onto a discontinuous CsCl gradient and centrifuged at 30,000 rpm for 5 hours at 4°C in a Beckman L-90 ultracentrifuge with an SW41Ti rotor (Beckman Coulter, Fullerton, USA). The banded bacteriophage particles were collected and thoroughly dialyzed against SM buffer. Final bacteriophage titers were determined using double agar overlay plaque assays (15). Purified bacteriophages were stored at 4°C and further diluted in SM buffer to achieve a working suspension of approximately 2×10^{10} pfu/ml prior to the assays.

Bacteriophage features

Morphology

The morphology of each of the five bacteriophages was analyzed by transmission electron microscopy at the Félix d'Hérelle Reference Center for Bacterial Viruses, Laval University, Québec, Canada. Briefly, a 200-mesh Formvar carbon-coated copper grid (Pelco International, Redding, USA) was deposited face down on 10 µl of staining suspension (2% uranyl acetate, pH 7.0, for all bacteriophages except for F770/05, which was stained with 2% phosphotungstic acid, pH 7.0). After 30 s, 10 µl of the bacteriophage suspension was mixed with the stain. After 2-3 minutes, the grid was deposited face up on blotting paper. The grid was dried for 5 minutes and then observed at 80 kV using a JEOL 1230

transmission electron microscope (JEOL, Peabody, USA). These data were integrated with the genomic analysis, and the bacteriophages were classified according to the Ackermann classification (16).

Genomic analysis

The DNA of all five bacteriophages was isolated using a standard phenol:chloroform extraction and DNA precipitation protocol (17). The purified nucleic acid was sent to Macrogen, Inc. (Seoul, Korea), for commercial sequencing. In brief, pyrosequencing of the sample DNA was performed using the GS FLX Titanium General Library Preparation Kit (Roche 454 Company, Branford, USA) according to the manufacturer's instructions. The assembly of quality-filtered reads was performed using Genome Sequencer De Novo Assembler software (Newbler) version 2.5.3. An extensive bioinformatics evaluation was conducted to analyze the sequences and identify regions of similarity with entries in databases, which yield clues about structure and function. Each genome sequence was scanned using the NCBI BlastN and BlastX bioinformatics tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Prediction of open reading frames (ORFs) was performed by integrating the results obtained by the programs GeneMark.hmm (http://exon.gatech.edu/genemark/eukhmm.cgi) and MetaGeneAnnotator (http://metagene.cb.k.u-tokyo.ac.jp). Protein homology searches were performed with the BlastP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) using the NCBI non-redundant protein sequence database. The genome sequences were deposited in the patent division of GenBank (specific patent numbers: WO2010090542 and WO2012036580).

Host range

The five bacteriophages were tested against a panel of clinical isolates using the bacteriophage spot-test procedure (18). Briefly, 3 ml of top-0.7% tryptone soy agar (TSA; Biokar Diagnostics, Pantin Cedex, France) was added to 200 µl of an overnight culture of each clinical isolate and poured over TSA. The agar was allowed to solidify, after which 5 µl of each bacteriophage suspension (approximately 10^8 pfu) was spotted on the bacterial lawn of each different isolate. The drop was allowed to dry, and the plates were incubated overnight at 37°C. Specific bacteriophage-sensitive isolates showed clear areas where the bacteriophage suspensions had been spotted.

Bacteriophage activity against planktonic cells

To determine the bacteriophages' activity against planktonic cells in vitro, a kinetic time-kill assay (19) was performed using a modified protocol. Briefly, 1 ml of the host bacterial suspension (5 × 10^8 cfu) was diluted in 9 ml of TSB, yielding a final concentration of 5 × 10^7 cfu/ml. For single-bacteriophage studies, 100 µl (5 × 10^9 pfu) of the specific bacteriophage was added, yielding a final concentration of 5 × 10^8 pfu/ml (bacteriophage-to-bacterium ratio or input multiplicity (IM) of 10). For combination studies, 100 µl (5 × 10^9 pfu) of each bacteriophage suspension was added, resulting in a final concentration of 5 × 10^8 pfu/ml (IM of 10) for each of the bacteriophages. An additional kinetic assay was performed for *P. aeruginosa* 433/07, in which 10 µl (5 × 10^8 pfu) of the bacteriophage F770/05 suspension was added (yielding an IM of 1), alone or in combination with the bacteriophage F510/08 at an IM of 10. Control experiments were performed in parallel using bacteriophage buffer instead of a bacteriophage suspension. All mixtures were incubated at 37°C with agitation, and 100 µl aliquots were collected at 0, 1, 3, 5, and 24 hours post-infection. Bacterial quantification was performed using the 10-fold serial dilution method (20). All experiments were conducted in triplicate. The results are presented as the mean ± standard deviation and are expressed as logarithm-transformed values (log (cfu/ml)) over time.

Combined bacteriophage activity against established biofilms

The bacteriophages' activity against established biofilms was examined using a modification of previously described protocols (21, 22). Briefly, 1 ml of each of the host bacterial suspensions (5 × 10^8 cfu) was diluted in 9 ml of TSB, and 100 µl of this dilution (5 × 10^6 cfu) was added to a 96-well flat-bottom polystyrene microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium) and incubated at 37°C for 24 hours to allow biofilm formation. After incubation, the planktonic bacteria were carefully removed with a sterile pipette. The number of biofilm cells at 24 hours has been previously demonstrated to be approximately 10^7 cfu/well for all bacterial species (12). Then, 150 µl of bacteriophage suspensions (IMs of 10 and 100) diluted in TSB was added to the wells. The following bacteriophage suspensions were used for each bacterium: for *S. aureus*, a 1:1 combination of F44/10 and F125/10; for *P. aeruginosa*, a combination of F770/05 and F510/08 at a 1:10 ratio; and for *A. baumannii*, F1245/05 alone. Biofilms treated with TSB alone served as positive controls in measurements of cell metabolic activity (see below).

The microplates were incubated at 37°C for either 4 or 24 hours. At each time point, the wells were processed according to a previously described protocol (21) using alamarBlue (AB; Thermo Scientific, Madrid, Spain), and their absorbances at 570 nm and 600 nm were measured using a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, USA). A second assay was performed in which, after biofilm formation, planktonic bacteria were removed from the wells and replaced with a bacteriophage suspension every 4 hours over a 24-hour incubation period. In the positive-control group, planktonic bacteria were removed from the wells and replaced with TSB every 4 hours. These plates were then processed as described (21).

Biofilm susceptibility experiments were performed a minimum of three times. All results are presented as the percent variation of AB ± standard deviation. This value was calculated using the manufacturer's formula, with one exception: the media-only negative control in the formula was replaced by a more robust negative control that consisted of media plus bacteriophage at each IM (*i.e.*, IMs of 10 and 100). Strong antimicrobial suppression was defined as a ≥ 50% reduction in AB compared with the positive control.

Statistical analysis

For all data sets, comparisons between groups were performed using two-tailed Student's *t* tests, and P-values < 0.05 were considered significant. All data were entered into a spreadsheet program (Excel, Microsoft, Redmond, USA) for statistical analysis. Analytical statistics were performed using Analyse-it, version 2.21 Excel 12+ (Analyse-it Software, Leeds, United Kingdom), a statistical add-in program for Excel.

Results

Bacteriophage features

After purification, the selected bacteriophages were initially characterized according to plaque morphology. The *S. aureus* F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 bacteriophages produced clear lytic plaques ranging from 1.5-5 mm in diameter. Plaques produced by the bacteriophages F770/05, F510/08, and F1245/05 were surrounded by growing opaque halo zones. The morphological and genomic characteristics of the five bacteriophages are presented in Figure B2.1-1.

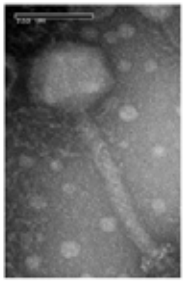
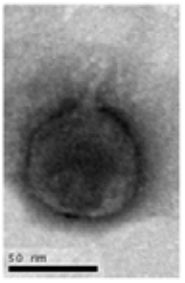
		Staphylococcus aureus		*Pseudomonas aeruginosa*		*Acinetobacter baumannii*
		F44/10	**F125/10**	**F770/05**	**F510/08**	**F1245/05**
Morphology	**ICTV classification**	*Myoviridae*		*Podoviridae*		
	electron micrograph					
		capsid 100±8 nm tail: 225±12 nm	capsid: 92±7 nm tail: 234±11 nm	capsid: 69±3 nm tail: 48±8 nm	capsid: 68±3 nm	capsid: 63±3 nm
Genome	**nucleic acid**	linear dsDNA	linear dsDNA	linear dsDNA	linear dsDNA	linear dsDNA
	size	137,360 bp	144,994 bp	72,177 bp	43,313 bp	43,016 bp
	homology group	*K-like*	*K-like*	*N4-like*	*ϕKMV-like*	undefined

Figure B2.1-1 – Morphological and genomic characteristics of the bacteriophages used for bacteriophage therapy. Five bacteriophages previously shown to successfully treat infections in vivo were characterized using transmission electron microscopy. Representative images are shown. The genomes were sequenced by pyrosequencing and analyzed extensively using BlastN, BlastX, GeneMark.hmm, MetaGeneAnnotator, and BlastP. ICTV = International Committee on Taxonomy of Viruses.

Morphology

To classify the purified bacteriophages based on their virion morphology, we used transmission electron microscopy. The staphylococcal bacteriophages F44/10 and F125/10 appeared to be composed of a contractile tail and an isometric head, with a baseplate structure also discernible at the tip of the F44/10 tail. These features, along with their genomic properties (see below), allowed us to classify F44/10 and F125/10 as *Myoviridae*. The *Pseudomonas* bacteriophages F770/05 and F510/08 and the *Acinetobacter* bacteriophage F1245/05 had short tails and were classified as *Podoviridae*. The *Podoviridae* family consists of different subgroups. Although there are certain morphological similarities between the bacteriophages F510/08 and F1245/05 and the ϕKMV-like group (23, 24), definite morphological assignment of F1245/05 could not be performed due to the uncharacteristic morphology of these virion particles.

Genomic analysis

Bacteriophages were characterized at the genomic level by determining and analyzing their genome sequences. The bacteriophages F44/10 and F125/10 had the largest genomes and hence a greater number of putative genes and ORFs, which is in agreement with the characteristic features of viruses belonging to the *Myoviridae* family (25). The genomes of the bacteriophages F44/10 and F125/10 displayed high similarity (up to 98% nucleotide sequence identity, 80-90% genome coverage) to those

of a group of highly related staphylococcal myoviruses, which includes bacteriophages K (26), A5W (27), and GH15 (28). The bacteriophages F510/08 and F770/05 shared high sequence identity (up to 98% nucleotide sequence identity, 83-98% genome coverage) with Pseudomonas ΦKMV-like and N4-like viruses, respectively (29). Examples of ΦKMV-like viruses are the bacteriophages ΦKMV and LUZ19 (30, 31) and of N4-like viruses are LIT1 and LUZ7 (29). The bacteriophage F1245/05 presented no significant similarity at the DNA level with any known bacteriophage in the databases, except for a few short segments with up to 4% nucleotide sequence identity and 81% genome coverage.

The deduced products of the predicted genes of all bacteriophages were compared with sequences in the NCBI non-redundant protein sequence database using BLASTP. No significant similarity with known virulence or toxin proteins or with elements typically associated with lysogeny (integrases, repressors, and antirepressors) could be found. Finally, the protein similarity searches did not reveal potential exopolysaccharide depolymerase genes.

Bacteriophage host range

To gain insight into the host range of selected bacteriophages, the susceptibility of three panels of clinical isolates of *S. aureus* (n = 132), *P. aeruginosa* (n = 93), and *A. baumannii* (n = 103) was tested for each species-specific bacteriophage. There was a degree of variability in the host range of each bacteriophage (Table B2.1-1). All tested staphylococcal strains were susceptible to both *S. aureus* bacteriophages (F44/10 and F125/10). In contrast, when examined individually, the *P. aeruginosa* bacteriophages F770/05 and F510/08 lysed only 63.4% and 68.8% of the tested isolates, respectively. However, when these results were considered together, we observed that 80.6% of the *P. aeruginosa* isolates were infected by at least one of the bacteriophages, whereas 51.6% were susceptible to both bacteriophages. Finally, of the tested *A. baumannii* strains, 74.8% were susceptible to the bacteriophage F1245/05.

Table B2.1-1 – Susceptibility of wound bacterial isolates to candidate bacteriophages for bacteriophage therapy.

	S. aureus		*P. aeruginosa*			*A. baumannii*
	F44/10	F125/10	F770/05	F510/08	F770/05 + F510/08	F1245/05
Number of bacterial strains tested	132		93			103
Bacterial strain's susceptibility	100%	100%	63.4%	68.8%	80.6%*	74.8%

*Percentage of *P. aeruginosa* isolates that were susceptible to at least one of the bacteriophages (only 51.6% of the isolates were susceptible to both)

Bacteriophage activity against planktonic cells

To evaluate the activity of the selected bacteriophages against planktonic cells, liquid cultures of the different bacterial hosts were exposed to the corresponding bacteriophages, both individually and in combination, and cell growth/viability was monitored over time with constant agitation. The time-kill curves are presented in Figure B2.1-2. *S. aureus* 743/06, when challenged with either F44/10 or F125/10 at an IM of 10, showed impaired growth, with reductions in cell counts of 2.3 ± 0.3 log (cfu/ml) and 2.2 ± 0.2 log (cfu/ml), respectively, 3 hours post-infection. However, after 24 hours, the

cultures recovered to near-control levels. The reduction in the number of viable cells observed at 3 hours was significantly enhanced when the two bacteriophages were used in combination (3.4 ± 0.2 log (cfu/ml); $P < 0.01$). Still, there was no difference in the recovery of growth at 24 hours.

At an IM of 10, the *P. aeruginosa* bacteriophage F510/08 caused a 3.9 ± 0.4 log (cfu/ml) reduction in the viability of *P. aeruginosa* 433/07 at 3 hours post-infection. This reduction was more modest (0.7 ± 0.4 log (cfu/ml)) for F770/05 at the same IM and time point. When the two bacteriophages were combined, the kill curve was not different from that of F770/05 for the first 5 hours; however, the combination provided a statistically significant reduction relative to the control at 24 hours (1.3 ± 0.3 log (cfu/ml); $P < 0.01$). When the IM of F770/05 was reduced to 1, combined with F510/08 at an IM of 10, the initial 3-hour reduction was more pronounced (2.5 ± 0.4 log (cfu/ml); $P < 0.01$). Similarly, this combination caused a statistically significant reduction (1.7 ± 0.3 log (cfu/ml); $P < 0.01$) relative to the control at 24 hours. *A. baumannii* 1305/05 suffered an initial 2.7 ± 0.2 log (cfu/ml) reduction at 3 hours after single-bacteriophage (F1245/05) challenge. Although this bacterial strain recovered by 24 hours, it did not reach control levels of viability.

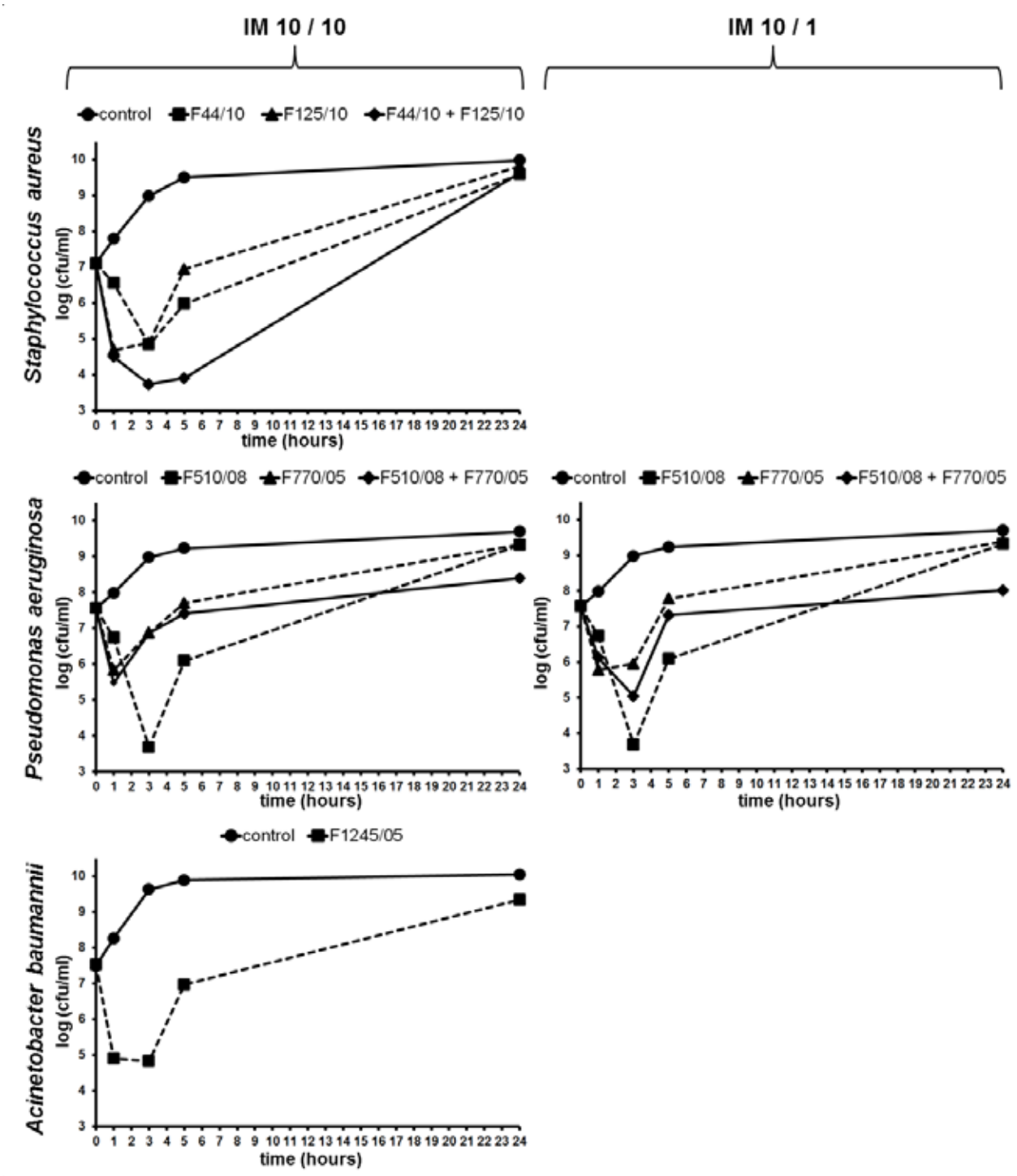


Figure B2.1-2 – Time-kill curves of the target bacteria during planktonic growth when challenged with their specific bacteriophages (alone or in combination). Bacterial strains were grown in TSB with constant agitation and with or without bacteriophages. Growth was monitored and quantified by calculating cfu/ml at 0, 1, 3, 5, and 24 hours. In the left panel (input multiplicity, IM 10/10), assays were performed on *S. aureus* 743/06, *P. aeruginosa* 433/07, and *A. baumannii* 1305/05 in which the specific bacteriophage suspensions were added to provide an IM of 10. In the right panel (IM 10/1), an additional assay was performed on *P. aeruginosa* 433/07 in which the bacteriophage F770/05 suspension was added to provide an IM of 1, alone or in combination with the bacteriophage F510/08 at an IM of 10.

Bacteriophage combinations' activity against established biofilms

We also studied the ability of the bacteriophages to eliminate cells in established biofilms by treating biofilms with species-specific bacteriophage combinations. AB, which quantitatively measures cell metabolic activity using an oxidation-reduction indicator that changes color in the presence of metabolically active cells, was used to measure cell viability in biofilms with and without treatment. The viability of cells within a biofilm is one of the most important aspects when evaluating the effectiveness of antimicrobial agents; therefore, we used a quantification method based on metabolically active cells, as determined by AB. This assay is a reliable and reproducible method for evaluating biofilm susceptibility and is considered to be preferable over the viable plate-count method, as it is very difficult to recover all surviving adherent bacteria as single cells using the latter method (21). The AB-based assay has been used to identify antimicrobials with enhanced efficacy against certain clinically important bacterial biofilms (21, 32).

Figure B2.1-3 shows the percentage of AB reduction in control and treated biofilms at 4 and 24 hours using different IMs and frequencies of application. At 4 hours, the tested bacteriophage preparations strongly reduced the cell viability of all bacterial hosts, independent of the IM. There was only a statistically significant difference between IMs for *A. baumannii*; the higher IM resulted in a greater reduction in metabolic activity (71.9 ± 5.8% vs. 88.7 ± 3.1%; $P < 0.01$).

At 24 hours, after a one-time bacteriophage preparation application, the cell viability of all bacterial strains was less suppressed than at 4 hours but still significantly different from that of the control. At 24 hours, there were no statistically significant differences between IMs except for *S. aureus,* for which the higher IM resulted in a greater reduction in cell viability (34.8 ± 8.5% vs. 52.6 ± 7.7%; $P < 0.01$). In experiments using multiple bacteriophage treatments, a greater reduction in cell viability was observed compared with the reduction following one-time bacteriophage treatment. This trend was found for *S. aureus* and *P. aeruginosa*, but not for *A. baumannii*.

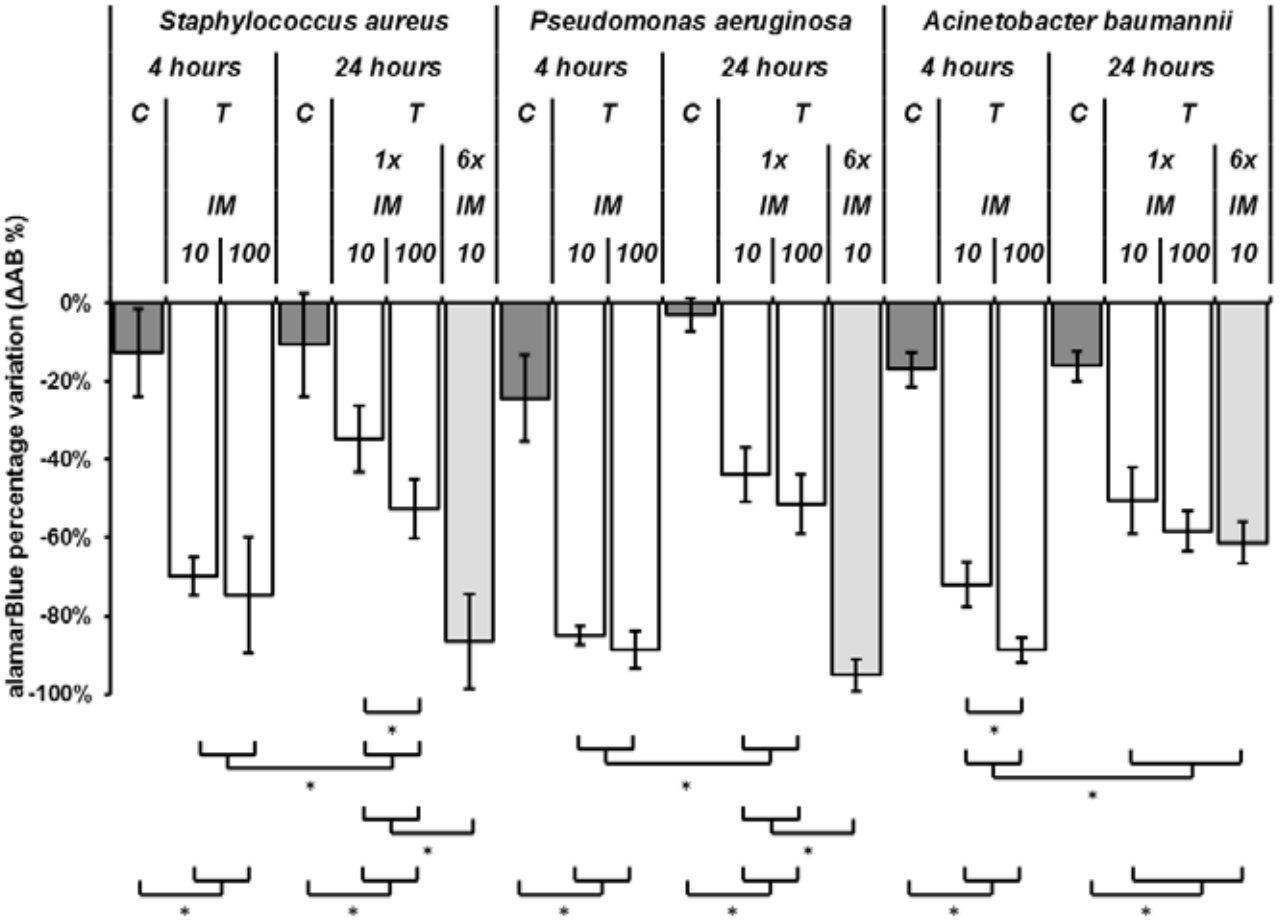


Figure B2.1-3 – Analysis of the bacteriophages' activity against bacterial biofilms. Bacteria were grown for 24 hours to establish biofilms, and bacteriophages were then added. The following bacteriophage suspensions were used for each bacterium: for *S. aureus*, a 1:1 combination of F44/10 and F125/10; for *P. aeruginosa*, a combination of F770/05 and F510/08 at a 1:10 ratio; and for *A. baumannii*, F1245/05 alone. Cell metabolism was quantified with alamarBlue (AB) and reported as the percent reduction relative to growth in untreated controls. C = negative control, T = treated with bacteriophage, IM = input multiplicity, 1x = one-time bacteriophage suspension application, 6x = bacteriophage suspension application every 4 hours for 24 hours. * $P < 0.01$ (dendritic lines establish the comparison between the different groups).

Discussion

Effective bacteriophage preparations for therapeutic purposes require careful design through a multi-step research process of bacteriophage characterization, cocktailing, and dosing. This process includes in vitro studies, such as those presented here, and in vivo studies, which have been previously published (11). Ideally, the characterization of bacteriophages for BT should be as thorough and complete as possible. However, in certain cases, it may be more practical to minimize this process and to focus the characterization on particular traits that are the most desirable for a specific application. Combining different bacteriophages in the same preparation (mixtures of two or more bacteriophages within a given formulation) frequently results in a broader spectrum of antibacterial activity and/or lytic efficacy and may allow targeting of bacteria under different conditions or in different environments (33). Finally, in vitro experiments such as those described in this work are useful for evaluating the direct interaction between a drug and bacteria, which enables

the selection of candidate bacteriophages. These studies also provide valuable information for the determination of optimal posology (34).

Integration of the information emerging from the morphological and genomic analyses revealed that the bacteriophages used here were all tailed bacteriophages (order *Caudovirales*), with two belonging to the *Myoviridae* family and three to *Podoviridae*. Genome sequence analysis did not identify any known genes related to lysogeny or traits that might enhance the virulence of the target bacteria, which is an important observation regarding the bacteriophages' safe use. Another important selection criterion for bacteriophages for BT is their host range, which should be as broad as possible, particularly including clinically prevalent bacterial species (35). In this study, members of *Myoviridae* exhibited the broadest spectrum of lytic activity, whereas the *Podoviridae* viruses exhibited a narrower spectrum, and particularly the pseudomonal bacteriophages. The spectrum of activity of the staphylococcal bacteriophages was relatively broad as expected, given their high relatedness to bacteriophages K and A5W, both of which have been previously described as polyvalent bacteriophages (36, 37). Still, the host ranges of the *Pseudomonas* and *Acinetobacter* bacteriophages are remarkable compared with those of other species-specific bacteriophages (38, 39). The overall morphology, genomic characterization, and host range results suggest that these bacteriophages are very good candidates for BT. However, care must be taken when generalizing these results, because the bacterial clinical isolates used for the bacteriophage host-range investigation reflect only the microbiological profile of diabetic foot ulcers in a particular geographical area, and these vary worldwide. Also the sensitivity of the spot test must be taken into account. While the use of high bacteriophage titers (10^8 PFU per spot) for host range analysis is routine when considering bacteriophages for BT (40), it should be noted that the use of lower titers may reduce host range. In our host-range investigation the use of bacteriophage stock dilutions up to 10^3 PFU per spot yielded differences from the presented results up to 23% (unpublished data).

Time-kill curves provide detailed information about antimicrobial efficacy against planktonic bacteria as a function of time. These curves are often used to study the antibacterial effect of single and combination drug compounds and dosing regimens before in vivo efficacy studies (19). In the current study, following bacteriophage exposure, all bacteria had an initial bacterial reduction to nadir between 1 and 3 hours post-infection, followed by regrowth that was noticeable after 5 hours and even more pronounced after 24 hours. The *Pseudomonas* bacteriophage combination resulted in a significantly greater reduction in bacteria compared with the reduction stimulated by most active single bacteriophage 24 hours after bacteriophage exposure. However, the decrease was insufficient to be considered as a synergic effect, defined as a ≥ 2 log (cfu/ml)-fold decrease by a combination compared with the most active single agent (19). In the *Pseudomonas* combination study, when an IM of 1 of the *Pseudomonas* F770/05 bacteriophage was used instead of an IM of 10, we observed greater initial bacterial reduction after 3 hours, but similar results were obtained at 24 hours. This interaction was not specifically analyzed in our study, and there is no obvious explanation for this, but clearly, further studies would be of interest.

The study presented here has certain limitations. First, only a single bacterial inoculum was used. This value was carefully selected based on several lines of evidence. A higher inoculum (10^7 cfu/ml) was used than the normal 10^5 cfu/ml inoculum used in previous time-kill studies testing antibiotics (19) because we wanted to mimic a worst-case scenario, similar to that found in wounds (41). In a previous epidemiological study (5), microbiological products (aspirates, biopsies or swabs collected using the Levine method) of clinically infected foot ulcers in patients with diabetes were found to have a maximum bioburden of 10^7 cfu/gram of tissue (or cm^2 of ulcer area). Additionally, the most

recent study using a previously optimized rodent model (11) tested this bacteriophage cocktail on infected wounds with a known average wound bioburden of 7.54 ± 0.19 log (cfu) per ulcer.

Second, the IM in nearly all experiments was 10 (fixed IM). The final chosen IM was selected based on the "multiplicity of 10 rule," which states that if the goal is significant reduction in bacterial density, then one should strive for on the order of 10 bacteriophages adsorbed to the average bacterium (42, 43). Previous studies on infected animal and human burn tissue have concluded that low-titer bacteriophage administration (IMs lower than 10) is unlikely to be successful (44, 45). Furthermore, increasing the IM increases the success of BT by reducing bacterial numbers.

Third, we observed regrowth in planktonic cells exposed to bacteriophages within 24 hours. This observation may be indicative of the development of resistance, as in vitro resistance is frequent in both BT and antibiotic therapy. For example, a study (46) previously found in vitro resistance frequencies of 10^{-6}-10^{-4} for single-phage treatments and 10^{-6} for double-phage or triple-phage cocktails against *Escherichia coli* O157:H7. Similarly, resistance to fusidic acid can readily be selected from an initial high inoculum, with an average frequency of 10^{-6}-10^{-8}.This resistance has not limited the antibiotic's topical use and does not appear to be a clinical problem (47, 48). However, these observations do not imply in vivo resistance. According to certain studies, the rate of the development of resistance to bacteriophages is approximately 10-fold lower than the rate of the development of antibiotic resistance (49). Nonetheless, as observed here, in vitro studies show that bacteriophage resistance can evolve within hours, independently of the use of bacteriophage combinations. However, the evolution of bacteriophage resistance in vitro does not seem relevant to in vivo scenarios, in which bacteria replicate more slowly and are challenged by more difficult environmental conditions. A previous study (50) found an average resistance frequency of 1.2×10^{-8} for *S. aureus* treated with bacteriophages in vitro. However, the researchers were unable to isolate any bacteriophage-resistant *S. aureus* strains in vivo. Indeed, even though the resistance of bacteria to the bacteriophage cocktails used here was not specifically studied, we previously found that the presence of residual bacteria did not globally hinder planimetric or histological improvement (11). In the current study, the greatest reduction in bacterial counts occurred at 3 hours, and regrowth was observed at 5 hours, which enabled us to conclude that the best time to give a "boost" application of bacteriophage would be between these two time points.

A previous study (unpublished data) found that the plaques of the bacteriophages F770/05, F510/08, and F1245/05 were surrounded by growing opaque halo zones, which could be related to the presence of a virion-associated exopolysaccharide depolymerase (51). This and related enzymes have been found to enhance the biofilm-eradicating activity of bacteriophages compared with non-depolymerase-producing bacteriophages (52). Based on genomic analysis, none of our bacteriophages seemed to produce any obvious extracellular polysaccharide or exopolysaccharide depolymerase. However, because bacteriophages that do not produce depolymerases have also been used in biofilm elimination (53), we sought to investigate the effect of bacteriophage combinations on the viability of target bacterial cells in preformed biofilms at 4 and 24 hours. Here, assays using an IM of 10 produced nearly identical results as assays using an IM of 100, with two exceptions. First, we observed different results between an IM of 10 and an IM of 100 after 4 hours for *A. baumannii*; however, this discrepancy may have arisen because only one bacteriophage was used. When previous experiments used a combination of two bacteriophages, the IM doubled, producing synergistic results (34). Second, differences between IMs were observed after 24 hours for *S. aureus*. This result may have occurred because the receptor for the bacteriophage F44/10, which we speculate to be N-acetylglucosamine in the cell-wall teichoic acid, is very frequent (relative to other receptors) in both live cells and bacterial

debris. This property means that active bacteriophages may adsorb to fragments of lysed cells, (debris) instead of live cells, at a higher rate. This phenomenon may ultimately lead to injection of the genetic material in a suicidal manner, eliminating the bacteriophage from the system (54). Increasing the IM amplifies the probability of bacteriophage-bacterium interaction, resulting in a true cell infection. Moreover, in vivo a bacteriophage dose sufficiently in excess of the target bacterium population (IM ≥100) should be given to account for bacteriophage loss, dilution (associated with absorption and distribution), decay and/or inefficiencies of bacteriophage adsorption to bacteria (e.g. inefficiencies in penetration into biofilms in vivo).It is well known that bacterial regrowth occurs after biofilms have been exposed to antibiotics (55). One possible way to limit this regrowth is through multiple dose applications. Our results using multiple dose applications, as opposed to single-application dosing, are similar to the results observed in Georgia, where BT is the current standard of clinical care, and in Poland, where BT is used as an experimental treatment under a compassionate-use regulatory provision (7, 56, 57). These results were also corroborated experimentally in previously published animal studies (50). This implies that a significant proportion of the bacteria in biofilm do not have genotypic resistance, but rather some form of phenotypic resistance, which is reversible by the modification of the causal environmental factors. Various equally valid and non-mutually excluding theories have been presented, that could explain the possible coexistence dynamics of bacteriophages and susceptible bacteria: numerical refuge, physiological refuge, and shielding by bacterial debris. The numerical refuge theory (58) predicts that simple mass-action interactions between bacteriophages and sensitive and resistant bacteria determine the stability of the population. So, in our study, when new bacteriophages were added (creating a higher bacteriophage density), a decline in the number of sensitive cells resulted. The physiological refuge hypothesis (59) postulates that during certain stages of bacterial life cycles sensitive bacteria may become transitorily resistant to bacteriophage infection. In the present study, fresh medium was then added. This altered the life cycle of the present sensitive bacteria (*e.g.*, from stationary to logarithmic), thereby potentially converting them from a temporarily resistant state into a susceptible state. Finally, the shielding by bacterial debris theory (54) predicts that active bacteriophages adsorb into fragments of lysed cells (debris) and inject their genetic material in a suicidal manner discounting from the system as a bacteriophage. In the present study, as new bacteriophages were added dead cells were removed, thus reducing nonproductive binding as described. None of these observations was noted for *A. baumannii*, perhaps because this was the only case in which we used a single bacteriophage, limiting the importance of non-heritable mechanisms in the reduction in resistance induced by mutation.

In conclusion, we prepared, purified, and characterized bacteriophage cocktails with a broad spectrum of activity against *S. aureus*, *P. aeruginosa,* and *A. baumannii* strains that commonly cause DFIs. The complementary studies on both planktonic cells and established biofilms allowed us to better evaluate the effects of a high IM (≥ 10) and a multiple-dose treatment protocol (every 4 hours for 24 hours). We believe that this work takes an important step toward the future clinical application of BT.

References

1. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004 May;27(5):1047-53.
2. Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA. Risk factors for foot infections in individuals with diabetes. Diabetes Care. 2006 Jun;29(6):1288-93.
3. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. Clin Infect Dis. 2004 Oct 1;39(7):885-910.
4. Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW. A review of the scientific evidence for biofilms in wounds. Wound Repair Regen. 2012 Sep-Oct;20(5):647-57.
5. Mendes JJ, Marques-Costa A, Vilela C, Neves J, Candeias N, Cavaco-Silva P, et al. Clinical and bacteriological survey of diabetic foot infections in Lisbon. Diabetes Res Clin Pract. 2012 Jan;95(1):153-61.
6. Ansaldi M. Cell biology perspectives in phage biology. Front Biosci (Elite Ed). 2012;4:1823-9.
7. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, et al. Phage therapy in clinical practice: treatment of human infections. Curr Pharm Biotechnol. 2010 Jan;11(1):69-86.
8. Bruynoghe R, Maisin J. [Essais de thérapeutique au moyen du bacteriophage]. C R Soc Biol. 1921;85:1120-1.
9. Chopra I, Hodgson J, Metcalf B, Poste G. The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. Antimicrob Agents Chemother. 1997 Mar;41(3):497-503.
10. Abedon ST. The 'nuts and bolts' of phage therapy. Curr Pharm Biotechnol. 2010 Jan;11(1):1.
11. Mendes JJ, Leandro C, Corte-Real S, Barbosa R, Cavaco-Silva P, Melo-Cristino J, et al. Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds. Wound Repair Regen. 2013 Jul;21(4):595-603.
12. Mottola C, Mendes J, Cavaco-Silva P, Melo-Cristino J, Oliveira M. Relevance of inoculum size on biofilm formation by diabetic foot bacterial isolates. Portuguese Congress of Microbiology and Biotechnology' 2013; Aveiro2013.
13. Adams MH. Methods of study of bacterial viruses: Isolation of bacterial viruses. Bacteriophages. 1st ed. New York, USA: Interscience; 1959. p. 447-9.
14. Miller H. Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. Methods Enzymol. 1987;152:145-70.
15. Kropinski A, Mazzocco A, Waddell T, Lingohr E, Johnson R. Enumeration of bacteriophages by double agar overlay plaque assay. In: Clokie M, Kropinski A, editors. Bacteriophages Methods and protocols, volume 1: isolation, characterization, and interactions. New York: Humana Press, Springer Science + Business Media; 2009. p. 69-76.
16. Ackermann H-W. Phage classification and characterization. In: Clokie M.R.J. and Kropinski A.M. e, editor. Bacteriophages Methods and Protocols. Clifton, NJ: Humana Press; 2009. p. 127-40.
17. Sambrook J, Fritsch E, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed ed. Cold Spring Harbor, NY: CSH Laboratory Press; 1989.
18. Armon R, Kott Y. A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. J Appl Bacteriol. 1993 Apr;74(4):490-6.
19. NCCLS. Methods for determining bactericidal activity of antimicrobial agents: approved guideline. 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087 USA: NCCLS; 1999.
20. Murray P, Baron E, Jorgensen J, Pfaller M, Yolken R. Manual of clinical microbiology. 8th ed. Washington, DC: ASM Press; 2003.

21. Pettit RK, Weber CA, Kean MJ, Hoffmann H, Pettit GR, Tan R, et al. Microplate Alamar blue assay for Staphylococcus epidermidis biofilm susceptibility testing. Antimicrob Agents Chemother. 2005 Jul;49(7):2612-7.
22. Cerca N, Martins S, Cerca F, Jefferson KK, Pier GB, Oliveira R, et al. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. J Antimicrob Chemother. 2005 Aug;56(2):331-6.
23. Chang KC, Lin NT, Hu A, Lin YS, Chen LK, Lai MJ. Genomic analysis of bacteriophage varphiAB1, a varphiKMV-like virus infecting multidrug-resistant Acinetobacter baumannii. Genomics. 2011 Apr;97(4):249-55.
24. Lammens E, Ceyssens PJ, Voet M, Hertveldt K, Lavigne R, Volckaert G. Representational Difference Analysis (RDA) of bacteriophage genomes. J Microbiol Methods. 2009 May;77(2):207-13.
25. Lavigne R, Darius P, Summer EJ, Seto D, Mahadevan P, Nilsson AS, et al. Classification of Myoviridae bacteriophages using protein sequence similarity. BMC Microbiol. 2009;9:224.
26. O'Flaherty S, Coffey A, Edwards R, Meaney W, Fitzgerald GF, Ross RP. Genome of staphylococcal phage K: a new lineage of Myoviridae infecting gram-positive bacteria with a low G+C content. J Bacteriol. 2004 May;186(9):2862-71.
27. Structural and functional characterization of polyvalent Staphyloccocal bacteriophage A5W [database on the Internet]2008.
28. Gu J, Liu X, Lu R, Li Y, Song J, Lei L, et al. Complete genome sequence of Staphylococcus aureus bacteriophage GH15. J Virol. 2012 Aug;86(16):8914-5.
29. Ceyssens PJ, Brabban A, Rogge L, Lewis MS, Pickard D, Goulding D, et al. Molecular and physiological analysis of three Pseudomonas aeruginosa phages belonging to the "N4-like viruses". Virology. 2010 Sep 15;405(1):26-30.
30. Ceyssens PJ, Lavigne R, Mattheus W, Chibeu A, Hertveldt K, Mast J, et al. Genomic analysis of Pseudomonas aeruginosa phages LKD16 and LKA1: establishment of the phiKMV subgroup within the T7 supergroup. J Bacteriol. 2006 Oct;188(19):6924-31.
31. Lavigne R, Burkal'tseva MV, Robben J, Sykilinda NN, Kurochkina LP, Grymonprez B, et al. The genome of bacteriophage phiKMV, a T7-like virus infecting Pseudomonas aeruginosa. Virology. 2003 Jul 20;312(1):49-59.
32. Pettit RK, Weber CA, Pettit GR. Application of a high throughput Alamar blue biofilm susceptibility assay to Staphylococcus aureus biofilms. Ann Clin Microbiol Antimicrob. 2009;8:28.
33. Chan BK, Abedon ST. Phage therapy pharmacology phage cocktails. Adv Appl Microbiol. 2012;78:1-23.
34. Abedon ST, Thomas-Abedon C. Phage therapy pharmacology. Curr Pharm Biotechnol. 2010 Jan;11(1):28-47.
35. Gill JJ, Hyman P. Phage choice, isolation, and preparation for phage therapy. Curr Pharm Biotechnol. 2010 Jan;11(1):2-14.
36. O'Flaherty S, Ross RP, Meaney W, Fitzgerald GF, Elbreki MF, Coffey A. Potential of the polyvalent anti-Staphylococcus bacteriophage K for control of antibiotic-resistant staphylococci from hospitals. Appl Environ Microbiol. 2005 Apr;71(4):1836-42.
37. Ulatowska M, Weber-Dabrowska B, Sadowy E, Krzyszton-Russjan J, Gorski A, Lobocka M. Structural and functional characterization of polyvalent Staphyloccocal bacteriophage A5W: Available from: http://www.ebi.ac.uk/ena/data/view/ACB89046.

38. Popova AV, Zhilenkov EL, Myakinina VP, Krasilnikova VM, Volozhantsev NV. Isolation and characterization of wide host range lytic bacteriophage AP22 infecting Acinetobacter baumannii. FEMS Microbiol Lett. 2012 Jul;332(1):40-6.
39. Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Tediashvili M, Lashkhi N, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One. 2009;4(3):e4944.
40. Kutter E. Phage host range and efficiency of plating. Methods Mol Biol. 2009;501:141-9.
41. Loc-Carrillo C, Wu S, Beck JP. Phage therapy of wounds and related purulent infections. In: Hyman P, editor. Bacteriophages in health and disease. 1st ed. Cambridge, USA: CAB International; 2012. p. 185-202.
42. Abedon ST. Kinetics of phage-mediated biocontrol of bacteria. Foodborne Pathog Dis. 2009 Sep;6(7):807-15.
43. Kasman LM, Kasman A, Westwater C, Dolan J, Schmidt MG, Norris JS. Overcoming the phage replication threshold: a mathematical model with implications for phage therapy. J Virol. 2002 Jun;76(11):5557-64.
44. Goode D, Allen VM, Barrow PA. Reduction of experimental Salmonella and Campylobacter contamination of chicken skin by application of lytic bacteriophages. Appl Environ Microbiol. 2003 Aug;69(8):5032-6.
45. Kumari S, Harjai K, Chhibber S. Topical treatment of Klebsiella pneumoniae B5055 induced burn wound infection in mice using natural products. J Infect Dev Ctries. 2010 Jun;4(6):367-77.
46. O'Flynn G, Ross RP, Fitzgerald GF, Coffey A. Evaluation of a cocktail of three bacteriophages for biocontrol of Escherichia coli O157:H7. Appl Environ Microbiol. 2004 Jun;70(6):3417-24.
47. Turnidge J, Collignon P. Resistance to fusidic acid. Int J Antimicrob Agents. 1999 Aug;12 Suppl 2:S35-44.
48. Sahm DF, Deane J, Pillar CM, Fernandes P. In Vitro Activity of CEM-102 (Fusidic Acid) Against Prevalent Clones and Resistance Phenotypes of S. aureus. Antimicrob Agents Chemother. 2013 Jun 17.
49. Carlton RM. Phage therapy: past history and future prospects. Arch Immunol Ther Exp (Warsz). 1999;47(5):267-74.
50. Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D. Experimental phage therapy against Staphylococcus aureus in mice. Antimicrob Agents Chemother. 2007 Aug;51(8):2765-73.
51. Cornelissen A, Ceyssens PJ, T'Syen J, Van Praet H, Noben JP, Shaburova OV, et al. The T7-related Pseudomonas putida phage phi15 displays virion-associated biofilm degradation properties. PLoS One. 2011;6(4):e18597.
52. Hughes KA, Sutherland IW, Jones MV. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiology. 1998 Nov;144 (Pt 11):3039-47.
53. Chibeu A, Lingohr EJ, Masson L, Manges A, Harel J, Ackermann HW, et al. Bacteriophages with the ability to degrade uropathogenic Escherichia coli biofilms. Viruses. 2012 Apr;4(4):471-87.
54. Rabinovitch A, Aviram I, Zaritsky A. Bacterial debris-an ecological mechanism for coexistence of bacteria and their viruses. J Theor Biol. 2003 Oct 7;224(3):377-83.
55. Kussell E, Kishony R, Balaban NQ, Leibler S. Bacterial persistence: a model of survival in changing environments. Genetics. 2005 Apr;169(4):1807-14.
56. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011 Mar;1(2):66-85.
57. Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Fortuna W, Letkiewicz S, Szufnarowski K, et al. Clinical aspects of phage therapy. Adv Virus Res. 2012;83:73-121.

58. Chao L, Levin BR, Stewart FM. A complex community in a simple habitat: an experimental study with bacteria and phage. Ecology. 1977;58:369-78.
59. Lenski RE, Levin BR. Constraints on the coevolution of bacteria and virulent phage: a model, some experiments and predictions for natural communities. Amer Naturalist. 1985 125(4):585-602.

Chapter 3: Rat model of diabetic wound infection

Specific framework

The clinical application of BT requires both in vitro and in vivo experimental validation. As experimental models, in vitro tests fail to reproduce the physiologic and pathogenic complexity of an organism, making in vivo models a crucial tool for ensuring clinical relevance (1). Therefore, when in vitro testing has shown the feasibility of a project and indicated the optimal dosage regimen of a treatment modality, the next logical step is to investigate the preparation using an animal model. Researchers have studied in vivo wound healing in a variety of species, including pigs (2), rabbits (3), rats (4-6) and mice (7, 8), by using different incisional, excisional, and granulation wound models in animals rendered diabetic through genetic modification or chemical induction. Animal models of infected cutaneous wounds have also been published (9, 10). In all these models, the kinetics of wound closure is evaluated by a combination of macroscopic and/or histologic data (Figure B3.0-1). The microbial burden may be evaluated by invasive or non-invasive methods. However, there is no appropriately standardized in vivo model to facilitate the screening of BT preparations or other topical antimicrobial therapy (TAT) agents and their impact on wound healing. None of the published models fully reproduces the current clinical practice treatment protocols of chronic wound infections, which include debridement, antimicrobials, and dressings (11). Mechanical debridement is particularly pivotal in this strategy because it converts the molecular and cellular environment of a chronic wound to that of an acute healing wound by removing scabs and debris (10, 12, 13). Sharp debridement, as elegantly demonstrated by Wolcott et al. (12), also opens a time-dependent therapeutic window for TAT. Thus, there is currently no clinical or experimental rationale for using TAT products in its absence. Hence, we needed to develop a new diabetic wound infection model optimized for testing TAT agents – namely BT preparations – accurately reproducing the pathophysiology of infected diabetic wound healing while using the current standard treatment, debridement.

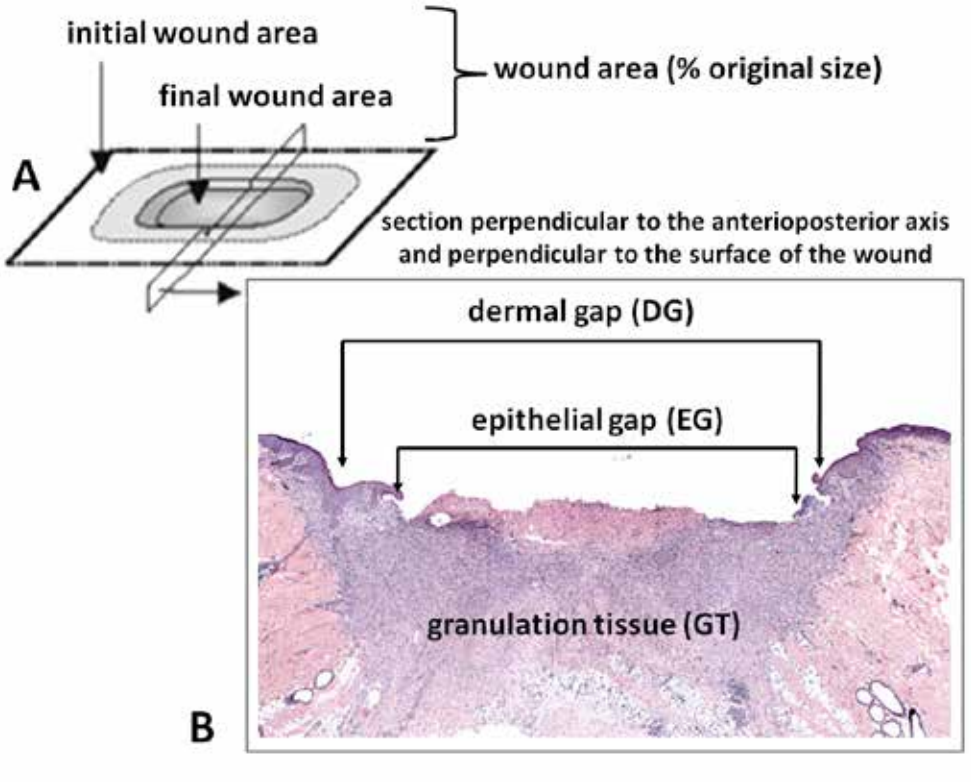


Figure B3.0-1 – Wound kinetics as quantified by planimetry and histology. (A) Planimetry: Wounds are photographed from standard height by using a mounted digital microscope in the beginning and at the end of the study, and wound area is expressed as a percentage of the initial wound area. (B) Histology: After animal euthanasia, harvested wounds can be examined histologically for both the epithelial gap (EG; distance between the advancing edges of clear, multiple-layer neoepidermis), dermal gap (DG; distance between uninjured dermis on both sides of the wound) and granulation tissue area (GT area; as calculated by computerized morphometric analysis).

References

1. Scherer SS, Pietramaggiori G, Mathews JC, Chan R, Fiorina P, Orgrill DP. Wound healing kinetics of the genetically diabetic mouse. Wounds. 2008;20(1):18-28.
2. Velander P, Theopold C, Hirsch T, Bleiziffer O, Zuhaili B, Fossum M, et al. Impaired wound healing in an acute diabetic pig model and the effects of local hyperglycemia. Wound Repair Regen. 2008 Mar-Apr;16(2):288-93.
3. Ahn ST, Mustoe TA. Effects of ischemia on ulcer wound healing: a new model in the rabbit ear. Ann Plast Surg. 1990 Jan;24(1):17-23.
4. Romana-Souza B, Nascimento AP, Monte-Alto-Costa A. Propranolol improves cutaneous wound healing in streptozotocin-induced diabetic rats. Eur J Pharmacol. 2009 Jun 2;611(1-3):77-84.
5. Toker S, Gulcan E, Cayc MK, Olgun EG, Erbilen E, Ozay Y. Topical atorvastatin in the treatment of diabetic wounds. Am J Med Sci. 2009 Sep;338(3):201-4.
6. El-Aziz A, El-Banna T, Abo-Kamar A, Ghazal A, AboZahra R. In vitro and in vivo activity of some antibiotics against staphylococcal biofilm and planktonic cells isolated from diabetic foot infections. Journal of American Science. 2010;6(12):760-70.
7. Braiman-Wiksman L, Solomonik I, Spira R, Tennenbaum T. Novel insights into wound healing sequence of events. Toxicol Pathol. 2007;35(6):767-79.
8. Tsuboi R, Rifkin DB. Recombinant basic fibroblast growth factor stimulates wound healing in healing-impaired db/db mice. J Exp Med. 1990 Jul 1;172(1):245-51.
9. Shi CM, Nakao H, Yamazaki M, Tsuboi R, Ogawa H. Mixture of sugar and povidone-iodine stimulates healing of MRSA-infected skin ulcers on db/db mice. Arch Dermatol Res. 2007 Nov;299(9):449-56.
10. Zhao G, Hochwalt PC, Usui ML, Underwood RA, Singh PK, James GA, et al. Delayed wound healing in diabetic (db/db) mice with Pseudomonas aeruginosa biofilm challenge: a model for the study of chronic wounds. Wound Repair Regen. 2010 Sep-Oct;18(5):467-77.
11. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. Clin Infect Dis. 2004 Oct 1;39(7):885-910.
12. Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, et al. Biofilm maturity studies indicate sharp debridement opens a time- dependent therapeutic window. J Wound Care. 2010 Aug;19(8):320-8.
13. Schultz GS, Sibbald RG, Falanga V, Ayello EA, Dowsett C, Harding K, et al. Wound bed preparation: a systematic approach to wound management. Wound Repair Regen. 2003 Mar;11 Suppl 1:S1-28.

A rat model of diabetic wound Infection for the evaluation of topical antimicrobial therapies.

Mendes JJ,Leandro C, Bonaparte D, Pinto A.
Comp Med. 2012 Feb;62(1):37-48.
PMID: 22330650 [PubMed - indexed for MEDLINE]

Abstract

Diabetes mellitus is an epidemic multisystemic chronic disease that frequently is complicated by complex wound infections. Innovative topical antimicrobial therapy agents are potentially useful for multimodal treatment of these infections. However, an appropriately standardized in vivo model is currently not available to facilitate the screening of these emerging products and their effect on wound healing. To develop such a model, we analyzed, tested, and modified published models of wound healing. We optimized various aspects of the model, including animal species, diabetes induction method, hair removal technique, splint and dressing methods, the control of unintentional bacterial infection, sampling methods for the evaluation of bacterial burden, and aspects of the microscopic and macroscopic assessment of wound healing, all while taking into consideration animal welfare and the '3Rs' principle. We thus developed a new wound infection model in rats that is optimized for testing topical antimicrobial therapy agents. This model accurately reproduces the pathophysiology of infected diabetic wound healing and includes the current standard treatment (that is, debridement). The numerous benefits of this model include the ready availability of necessary materials, simple techniques, high reproducibility, and practicality for experiments with large sample sizes. Furthermore, given its similarities to infected-wound healing and treatment in humans, our new model can serve as a valid alternative for applied research.

Introduction

The world is facing a growing diabetes mellitus (DM) epidemic, and diabetic foot ulcers, with an estimated lifetime risk of 25%(1), constitute one of the most common complications of this disease. About 58% of ulcers become clinically infected (2), often leading to amputation. Diabetic wounds (3, 4) do not follow the precisely orchestrated course of events observed in normal healing, and bacterial colonization or infection further disrupts this process (5). In current clinical practice, once an adequate blood supply is assured, the treatment of diabetic foot infections includes debridement, systemic antibiotics, and dressings (6). Mechanical debridement is pivotal to this strategy, because it not only significantly reduces the bioburden but also opens a time-dependent therapeutic window for topical antimicrobial therapy (TAT) (7). TAT agents consist of an active antimicrobial molecule associated with a vehicle or base, thereby delivering a high and sustained antibiotic concentration directly to the site of infection while avoiding systemic toxicity (8).

Several new TAT agents are under investigation, and a phased strategy of efficacy and toxicity testing is required before they can be made widely available for human use. None of the presently available in vitro or in vivo models of wound healing is optimal. As experimental models, in vitro tests fail to reproduce the physiologic and pathogenic complexity of an organism, making in vivo models a crucial tool for ensuring clinical relevance (9). Researchers have studied in vivo wound healing in a variety of species, including pigs (10), rabbits (11), rats (12-14) and mice (3, 15), by using different incisional, excisional, and granulation wound models in animals rendered diabetic through genetic modification or chemical induction.

For most researchers, rodents are the model of choice because they are inexpensive, easy to handle, require little space, and have accelerated healing compared with humans, thereby yielding for faster results (9). The excisional wound model accommodates the broadest assessment of the mechanisms involved in wound healing, including epithelialization, granulation, and angiogenesis (16). In addition, this model supports the evaluation of new topical pharmacologic interventions because medications can be applied directly to the wound bed (13, 15). All models of excisional wound healing are based on the same principles (Figure B3.2-1) (9). However, these models have been criticized because the main mechanism of wound healing in rodents is contraction (17) due to the presence of the panniculus carnosus muscle in the subcutaneous tissue; in contrast, humans heal more through reepithelialization (5). One rodent excisional wound healing model minimizes wound contraction by the use of silicone splints that are fixed to the skin by using immediate-bonding adhesive and nylon sutures (17). Although potentially useful for evaluating diabetic wound bacterial infections, this model has not been tested or validated in this context. Other published animal models of infected cutaneous wounds (18, 19) fail to afford the optimal characteristics of the model cited previously (17). Still, this model (17) is not without disadvantages, namely the difficulty of applying and maintaining the dressing (and splint) while maintaining stringent infection control.

Because, to our knowledge, better in vivo models are not available for the evaluation of TAT in diabetic wound infections, we refined and adapted the previous murine model (17) to develop a useful and cost-effective model for this purpose.

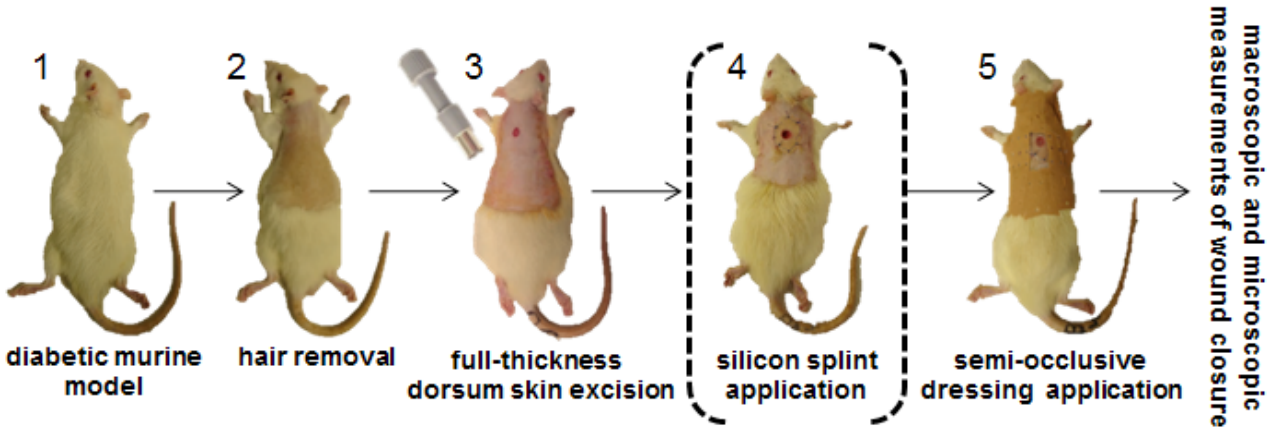


Figure B3.2-1 – Schematic representation of diabetic rodent models of excisional wound healing. **(1)** Genetically modified or chemically induced diabetic animals are used. **(2)** Hair is removed (various techniques are available). **(3)** A full-thickness wound extending through the panniculus carnosus is created in the interscapular region of the upper back, typically by using a punch biopsy instrument. **(4)** Some models use a silicone splint fixed to the skin, for minimizing wound contraction while allowing the normal granulation and reepithelialization. **(5)** The wound is covered with a semiocclusive dressing. Various macroscopic and microscopic methods are available for the evaluation of wound closure.

Materials and methods

This study was approved locally by the Animal Ethics Committee of the Instituto de Medicina Molecular and nationally by the Portuguese General Directorate of Veterinary Services (Direcção Geral de Veterinária), in accordance with Portuguese law. All animals were maintained in accordance with European Directive 86/609/EC (20), Portuguese law (Portaria 1005/92) (21), and the *Guide for the Care and Use of Laboratory Animals* (22). This study included the refinement and optimization of several sequential procedures. The animals used and the DM induction protocol were the same for all study groups. The final optimized wound-infection model study was preceded by 3 sequential optimization studies: hair removal (optimization study 1); prevention of unintentional critical colonization (optimization study 2); and assessment of wound bioburden (optimization study 3). To reduce the number of study groups, and therefore the total number of animals, each subsequent optimization study incorporated the findings of previous studies.

Animals

Specific pathogen-free male Wistar rats (Crl:WI[Han]; weight, 250 to 350 g; age, 8 to 10 wk) were obtained from Charles River Laboratories (L' Arbresle Cedex, France) and kept in an approved animal care center. The rats were maintained in microisolation caging in a room with controlled humidity (50% to 70%) and temperature (20 to 22 °C), a 14:10-h light:dark cycle, and free access to pelleted rodent chow (RM3, Special Diet Systems, Essex, UK) and filter-sterilized water. Initially housed in groups of 2, rats were housed individually after hair removal to preserve skin and dressing integrity.

Induction of DM

DM was induced chemically as described previously (23). Briefly, after a 12-h fast, rats received a single intraperitoneal injection of streptozotocin (65 mg/kg; Merck Chemical, Darmstadt, Germany) freshly prepared in 0.1 M sodium citrate buffer (pH 4.5). At 8 d after streptozotocin injection, blood glucose measurement was performed on tail-vein blood by using a glucometer (Accu-Chek Aviva

Nano, Roche Diagnostics, Penzberg, Germany). Rats whose fasting blood glucose levels exceeded 250 mg/dL (13.9 mmol/dL) were considered diabetic. Water intake and weight were monitor throughout the study, and to confirm the diabetic state, fasting blood glucose measurement was repeated on the day of euthanasia.

Optimization study 1: hair removal

On the day of DM confirmation, 9 diabetic rats were anesthetized by intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (25 mg/kg), and their dorsal surface hair was trimmed with an electric clipper. Rats then were divided into 3 groups depending on the method used to remove any remaining hair: straight razor; 2) depilatory cream (Opilca, GlaxoSmithKline Consumer Healthcare, Copenhagen, Denmark); and 3) cold wax (Veet cold wax strips, Reckitt Benckiser, West Ryde, Australia). The dorsum of all rats was rinsed with a 10% povidone-iodine solution and, after drying and cleansing, a liquid film-forming acrylate (Cavilon Skin Cleanser, *3M* Health Care, Saint Paul, MN) was applied evenly to cover the hair removal area. A photograph of the dorsum of the rat was taken from a 1.5 cm standard height (ES65 digital camera, Samsung, Beijing, China), and the rats were placed on a 37 °C heating pad to minimize hypothermia. All rats received sterile sodium chloride to prevent dehydration. After fully recovering from anesthesia, rats were placed in individual cages. Photographs of nonanesthetized rats were taken on days 4 and 14 after hair removal and used by 3 independent observers for evaluation of the hair-density index (scale: 1 [no hair] to 5 [normal amount of hair]) and skin-damage index (scale: 1, intact skin; 2, erythematous skin; 3, epidermal injury; 4, dermal injury; and 5, subcutaneous layer injury). Hair density and skin damage scores are expressed as the median (first and third quartiles).

Optimization study 2: prevention of unintentional critical colonization

Based on the findings of the previous optimization study, 18 Wistar rats with chemically induced DM were epilated by using cold wax and anesthetized by intraperitoneal injection of xylazine-ketamine 4 d thereafter.

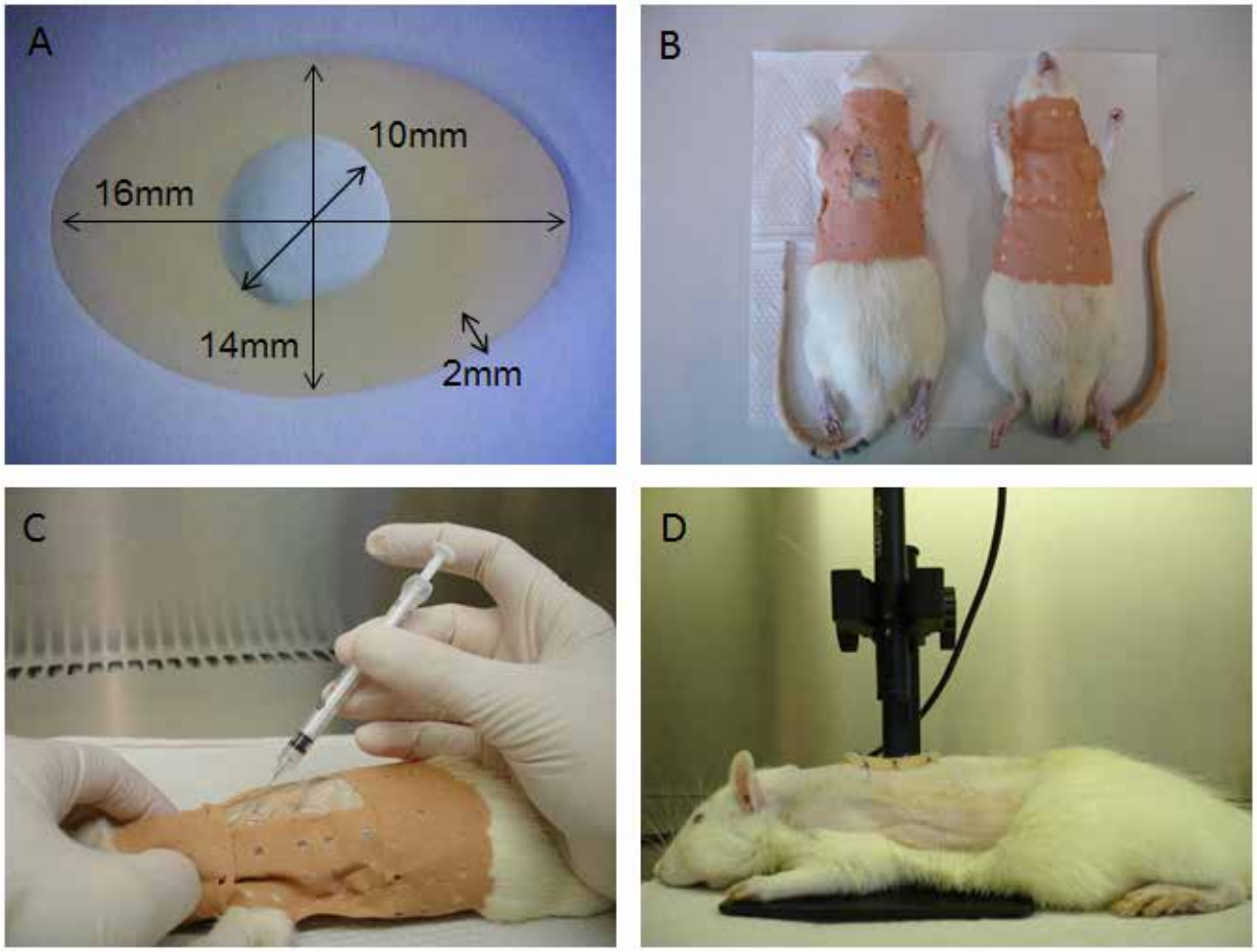


Figure B3.2-2 – Illustration of specific techniques. **(A)** The oval-shaped silicone splint and its dimensions. **(B)** Application of a jacket made from adhesive tape to prevent dressing loss. **(C)** Bacterial inoculation of the wound bed by inserting a 27-gauge, 19-mm needle attached to a 1-mL disposable syringe through the silicon splint at a 45° angle. **(D)** Wounds photographed from a standard 1.5 cm height by using a mounted digital microscope.

Decontamination protocols

All surgical procedures were performed in a sanitized surgery room by using autoclave-sterilized instruments. Because the procedures were repeated in multiple rats, 2 sets of instruments were used. Between uses, instruments were cleaned thoroughly to remove all organic debris, disinfected with a multipurpose disinfectant (Virkon, Antec International Limited, Suffolk, UK), and resterilized by using a glass bead sterilizer (FST 250, Fine Science Tools, North Vancouver, Canada) according to the manufacturer's guidelines. The surgeon wore clean scrubs, mask, and hair cap and used fresh sterile gloves for each rat.

Wounding, splinting, and dressing

The anesthetized rats were separated into 3 groups depending on the method of decontamination of the dorsal skin: thorough washing with sterile saline only; thorough washing with sterile saline followed by disinfection with 10% povidone-iodine; and sterile saline plus povidoneiodine (10 min contact time) followed by 70% isopropyl alcohol. A punch biopsy instrument (diameter, 6 mm; Accu-Punch, Acuderm, Fort Lauderdale, USA) then was used to create a full-thickness round wound extending through the panniculus carnosus in the interscapular region of the upper back of each rat, and the skin flap was excised by using iris scissors. An oval-shaped silicone splint (Figure B3.2-2

A) was adapted from a self-adhesive corn cushion (Comforsil, Toledo, Spain). Immediate-bonding cyanoacrylate glue in a disposable single-dose package (Loctite, Henkel Corporation, Westlake, OH) was used to fix the splint to the skin, followed by interrupted 3-0 nylon sutures to ensure its position. Liquid film-forming acrylate was applied to the epilated area, and the wound and surrounding area were covered with a previously tailored, semiocclusive, nonwoven polyester dressing (Fixomull Stretch, BSN Medical, Hamburg, Germany). The splint and dressing were maintained in place throughout the entire course of the experiment by the use of a jacket (Figure B3.2-2 B) made from adhesive tape (Leukoplast surgical tape, BSN Medical).

Debridement of the ulcer and assessment of contamination

Four days after wounding, the semiocclusive dressing was removed, and a scab, defined as a crust of dried blood, serum, and exudate (19), was noted over each wound. By using strict aseptic technique, the ulcer was debrided by simple mechanical removal of the scab. The ulcer then was photographed as previously described, and a liquid Amies elution swab (eSwab Collection and Preservation System, Copan, Corona, CA) was used to collect and transport swab cultures. Bacteria collection was performed by using the one-point method (24). Briefly, by using the sterile swab, the center surface of each wound was scrubbed carefully by rotating the swab 3 times clockwise with enough manual pressure to produce a small amount of exudate. The inoculated swab was inserted into a tube containing 1 mL liquid Amies transport medium and transported to the laboratory for immediate processing. The swab collection tube was vortexed (with the swab inside) for 5 s, and a 100-µL aliquot of the suspension was used for serial dilutions. Quantification of the viable bacteria present in the swab was performed by using the 10-fold serial dilution method (25), and 100 µL of each dilution was plated onto tryptone soy agar (Biokar Diagnostics, Pantin Cedex, France). The plates were incubated under aerobic conditions at 37 °C for 24 h, after which colonyforming units were counted. The wound and surrounding area again were covered with a previously tailored, semiocclusive, nonwoven polyester dressing. Because all of these procedures are considered to be painless and noninvasive, they were performed on unanesthetized rats. A surgical drape was placed over the head of the rat to reduce stress and ensure immobilization. The entire procedure (including debridement) was repeated on days 5, 8, and 11. Rats were evaluated twice daily for the integrity of the adhesive jacket, which was reinforced whenever deemed necessary. On day 11, the rats were euthanized by intraperitoneal injection of pentobarbital (200 mg). Quantitative microbiologic results were expressed as the number of colony-forming units per swab and, on the basis of previous studies (26) and the results of optimization study 3, rats with more than 10^3 cfu/swab on any given day were considered to be critically colonized. Results are presented as unit values and percentages.

Optimization study 3: assessment of wound bioburden

For this experiment, 14 Wistar rats with chemically induced DM were epilated by using cold wax, and an incision was made in each rat, splinted, and dressed as previously optimized and described. These ulcers were inoculated with bacterial suspensions of either *S. aureus* or *P. aeruginosa.* Clinical strains of *S. aureus* and *P. aeruginosa* that previously had been isolated from patients with chronic skin ulcers and cryopreserved at −70°C were grown on tryptone soy agar. After 24 h, bacterial cultures were harvested, and a bacterial suspension was prepared and compared with a McFarland standard (bioMérieux, Craponne, France). The inoculation dose was approximately 2.0 × 107 cfu/mL. The rats were divided into 2 groups of 7 animals each (*S. aureus* and *P. aeruginosa*). After application of the dressing and with the rat still anesthetized, the wound bed was inoculated with

100 µL of cultured bacteria (approximately 2.0 × 106 cfu) resuspended in sterile saline by inserting a 27-gauge, 19-mm needle attached to a 1-mL disposable syringe through the silicon splint at a 45° angle (Figure B3.2-2 C).

At 4 d after wounding, rats were euthanized by intraperitoneal injection of pentobarbital (200 mg), the semiocclusive dressing was removed, the ulcer was debrided, and a swab was obtained as described previously. Then, by using sterile surgical scissors, each wound was harvested in its entirety and placed in a sterile tube. Swabs and tissue samples were transported to the laboratory for immediate processing. The swabs were processed as previously described, and tissue samples were homogenized in 5 mL sterile saline in a pearl jar, vortexed for 20 s, and sonicated for 90 s at 35 MHz (Transsonic T570, Elma, Singen, Germany) to disaggregate bacteria (this procedure had been optimized previously to minimize cell disruption). A 100-µL volume of the homogenate was used for the serial dilutions. Quantification was performed by using the 10-fold serial dilution method (25); 100 µL of each dilution (either swab or tissue) was inoculated onto selective media (Chapman mannitol salt agar [Biokar Diagnostics] for *S. aureus* or cetrimide agar [Merck Chemical] for *P. aeruginosa*). The plates were incubated under aerobic conditions at 37 °C for 24 h, after which colony counts were performed. The isolates grown on Chapman mannitol salt agar were presumptively identified as *S. aureus* based on colony morphology and mannitol salt agar fermentation (27). The isolates grown on cetrimide agar were presumptively identified as *P. aeruginosa* based on colony morphology (28). Quantitative results are presented as the mean and standard deviation and expressed as logarithm-transformed values (log[cfu/swab] for swab samples and log[cfu/ulcer] for tissue samples). The data were compared by using a logarithmic scale owing to the wide variations in number of colony-forming units between cultures. Correlations were evaluated by the Pearson correlation coefficient, and the Fisher r-to-z method was used to calculate *P* values. A *P* value of less than 0.05 was considered significant. All data was entered into a spreadsheet program (Excel, Microsoft, Redmond, WA) for statistical analysis. Analytical statistics were performed by Analyse-it version 2.21 Excel 12+ (Analyse-it Software, Leeds, UK), a statistical add-in program for the spreadsheet program.

Optimized final rodent wound-infection model

General protocol and wound-closure kinetics

After every step of the procedure had been optimized, a final study (Figure B3.2-3) was designed by using 36 Wistar rats with chemically induced DM that were epilated by using cold-wax strips. Incisions were made, splinted, and dressed as previously described. Wounds were photographed from a 1.5-cm standard height by using a mounted digital microscope (SuperEyes 200× USB Digital Microscope, Shenzhen Tak and Assistive Technology, Shenzhen, China; Figure B3.2-2 D), and the rats were divided randomly into 3 groups: inoculated with *S. aureus* (*n* = 12), inoculated with *P. aeruginosa* (*n* = 12), and negative control (*n* = 12). The ulcers of the animals in the infected groups were inoculated with either *S. aureus* or *P. aeruginosa* as previously described, whereas the ulcers of the negative control group were inoculated with sterile saline. On day 4 after wounding, the semiocclusive dressing was removed, the ulcer debrided and photographed, and a swab was obtained as previously described. The entire procedure, including debridement, was repeated on days 5, 8, and 11, and the splint chamber was filled twice daily with sterile saline from day 5 until the end of the study. Wound kinetics were quantified by using image-processing software (ImageJ, US National Institutes of Health, Bethesda, MD) to measure the wound area by planimetry; wound area was expressed as a percentage of the initial wound area. Results are expressed as the mean of the percentage in area of the original wound size. Comparisons between groups were performed

by using 2-tailed Student *t* tests, and a *P* value of less than 0.01 was considered significant. All data were entered into the spreadsheet program, and analytical statistics were performed as described previously.

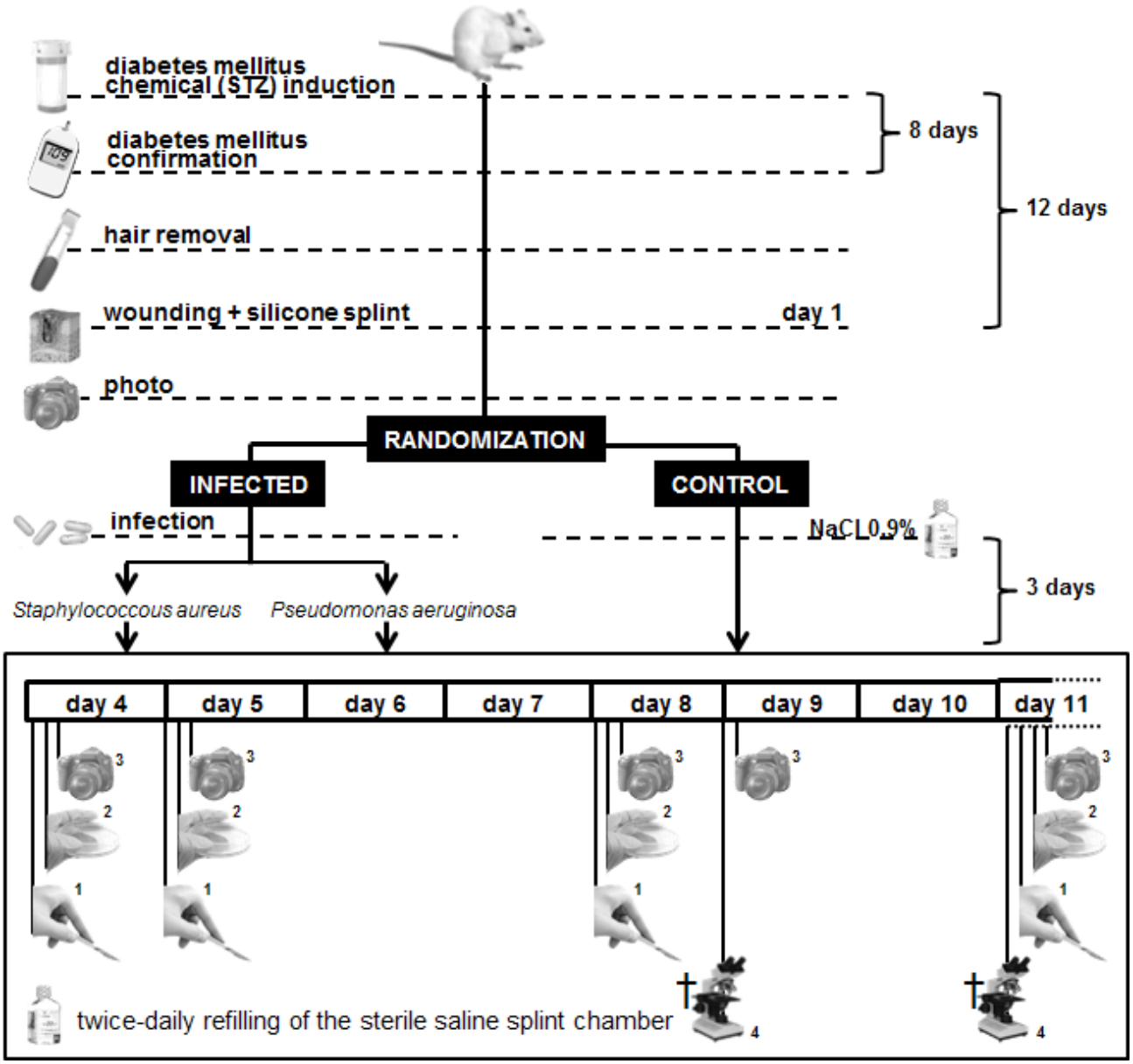


Figure B3.2-3 – Final study design: optimized rodent wound infection model. Wistar rats each received a single intraperitoneal injection of streptozotocin; 8 d after injection, rats considered to be diabetic (fasting blood glucose greater than or equal to 250 mg/dL) were epilated by using cold-wax strips. At 4 d after epilation (study day 1), skin was disinfected, wounded, splinted, and photographed from a standard height. In addition, rats were divided randomly into 2 infected groups (inoculated with either *S. aureus* or *P. aeruginosa*) and 1 negative control group (inoculated with sterile saline). On study days 4, 5, 8, and 11, ulcers were (1) debrided, (2) swabbed, and (3) photographed. From day 5 until the end of the study, splint chambers were filled twice daily with sterile saline. On study days 9 and 11, half of the animals in each group were (4) euthanized, and each ulcer was processed for histologic analysis.

Histologic analysis

For each group, 6 rats each were euthanized by intraperitoneal injection of pentobarbital (200 mg) on days 9 and 11, and each ulcer (including a 0.5-cm skin border) was harvested in its entirety by using sterile surgical scissors and placed in a tube. The sample was fixed overnight in 10% buffered formalin solution, after which the tissue was trimmed and cut through at the widest margin, embedded in paraffin, and sectioned in 3-µm increments. Sections were made perpendicular to the anterioposterior axis and perpendicular to the surface of the wound. For each wound, 2 serial sections were placed on a slide and stained with hematoxylin and eosin. Under light microscopy, the sections were photographed by using an upright brightfield microscope equipped with a color camera (model DM2500, Leica Microsystems, Wetzlar, Germany) at 50× magnification. Panoramic cross-sectional digital images of each wound were prepared by using professional image-editing software (Photoshop CS2, Adobe Systems, San Jose, CA). The images were analyzed for epithelial gap (EG), dermal gap (DG), and total granulation tissue (GT) area by using image-processing software. EG was defined as the distance between the advancing edges of clear, multiple-layer neoepidermis (9, 17), and its size was measured in millimeters, with an EG of 0 representing a completely reepithelialized wound. DG was defined as the distance between uninjured dermis on both sides of the wound (9, 17) and was measured in millimeters. GT area was calculated by computerized morphometric analysis (17) and expressed in square millimeters. The results are presented as mean ± SEM. Comparisons between groups were performed by using 2-tailed Student *t* tests, and a *P* value of less than 0.05 was considered significant. All data were entered into the spreadsheet program and analytical statistics were performed as described previously.

Results

Animals and induction of DM

We induced diabetes in 98 male Wistar rats with a success rate of 81.6%. Of the 80 successfully induced rats, 3.8% died prior to the end of the study; at necropsy, no significant gross pathology was noted. There was no mortality during the optimized final study. For the final study, 77 animals (78.6% of the number of rats induced initially) were used. At the time of euthanasia, all rats were confirmed to have fasting glucose levels of at least 250 mg/dL, and the mean weight loss was 18.10% ± 2.35%.

Optimization study 1: hair removal

Depilation by using a straight razor gave the worst hair density and skin damage results throughout the 15 d of the experiment (Figure B3.2-4). Although cold wax tended to cause increased immediate (day 1) epithelial injury, as measured by the skin-damage index, compared with that from the depilatory cream, this pattern was completely reversed by day 4. Epilatory hair removal by using cold wax achieved more prolonged results than did the depilatory methods (straight razor or depilatory cream), as evaluated by using the hair-density index, with less skin damage after day 4, which was the optimal day for infection.

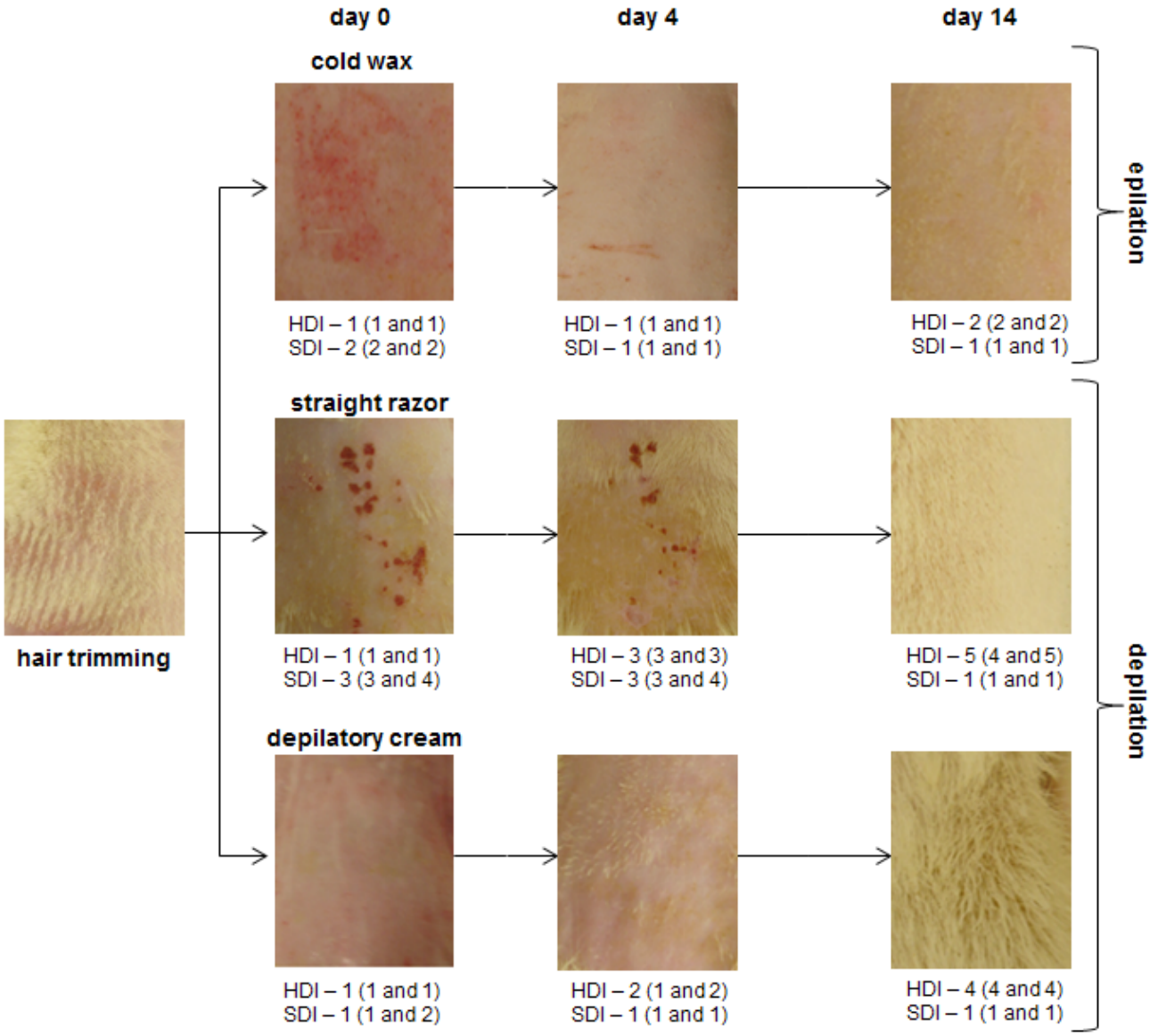


Figure B3.2-4 – Results of the hair-removal optimization study. After dorsal hair trimming, rats were divided into 3 groups: straight razor, depilatory cream, and cold wax. Photographs were taken immediately, 4 d, and 14 d after removal procedures; 3 independent observers evaluated 2 parameters: hairdensity index (HDI) and skin-damage index (SDI). Results are expressed as median (first and third quartiles).

Optimization study 2: prevention of unintentional critical colonization.
This experiment examined methods for preventing unintentional critical bacterial colonization. The use of a combined approach (washing with sterile saline, followed by disinfection with povidone-iodine and washing with isopropyl alcohol after 10 min contact time) produced the best results (Table B3.2-1), with only 1 of the 6 control wounds having a swab colony count that exceeded 3 logs (4.6×10^5 cfu/swab). No dressing or splint was lost during the experiment.

Table B3.2-1 – Evaluation of 3 strategies for preventing unintentional critical colonization (bacterial count >10^3 cfu/swab on any given study day). Note that the combined approach (washing with sterile saline, followed by disinfection with povidone-iodine and washing with isopropyl alcohol after 10 minutes contact time) produced the best results.

unintentional bacteria count	sterile saline[a] (n = 6)	sterile saline + povidone-iodine[b] (n = 6)	sterile saline + povidone--iodine + isopropanol[c] (n = 6)
≤ 10^3 (3 log)	2 (33.3 %)	3 (50.0 %)	5 (83.3 %)
> 10^3 (3 log)	4 (66.7 %)	3 (50.0 %)	1 (16.7 %)

[a] washed with sterile saline

[b] washed with sterile saline followed by disinfection with 10 % povidone-iodine

[c] washed with sterile saline followed by disinfection with 10 % povidone-iodine and washing with 70 **%** isopropanol after 10 minutes contact time

Optimization study 3: assessment of wound bioburden

The average colony count in swab samples (Table B3.2-2) was similar between the 2 species: 5.67 ± 0.16 log(cfu/swab) for *S. aureus* and 5.65 ± 0.26 log(cfu/swab) for *P. aeruginosa*. The colony counts in tissue samples differed between the 2 species by 0.16 log(cfu/ulcer) but this difference was not statistically significant (2-tailed *t* test, data not shown). The one-point quantitative swab method underestimated the number of bacteria in the wound bed by 1.79 ± 0.10 log(cfu) for *S. aureus* and 1.97 ± 0.16 log(cfu) for *P. aeruginosa*. The Pearson correlation coefficient (*r*) between swab and tissue colony counts was 0.810 (95% confidence interval, 0.143 to 0.971; *P* = 0.025) for *S. aureus* and 0.780 (95% confidence interval, 0.065 to 0.966; *P* = 0.037) for *P. aeruginosa*, suggesting a strong relationship between the 2 independent measures. This statistical correlation was not observed when data from the 2 infected groups were combined.

Table B3.2-2 – Bacterial colony counts (log[CFU]) from tissue or swab culture of wounds infected with *S. aureus* or *P. aeruginosa*. The variation between the two techniques (Δ swab/ulcer) is also presented. Note there was a statistical correlation for the individual groups but not when both of the infected groups were combined.

	ulcer log(CFU/ulcer)	**swab log(CFU/swab)**	**Δ swab/ulcer log(CFU)**	**R**	***P*[a] (95 % CI)**
S. aureus (n = 7)	7.46 ± 0.17	5.67 ± 0.16	1.79 ± 0.10	0.810	0.025 (0.143-0.971)
P. aeruginosa (n = 7)	7.62 ± 0.18	5.65 ± 0.26	1.97 ± 0.16	0.780	0.037 (0.065-0.966)
TOTAL (n = 14)	**7.54 ± 0.19**	**5.66 ± 0.21**	**1.88 ± 0.16**	**0.743**	***N.S.***

[a] *P* value as calculated by the Fisher r-to-z method

Optimized rodent wound-infection model

Wound-closure kinetics

In the final wound-infection model study, the control group showed continuous reduction of the wound area that was enhanced after mechanical debridement (Figure B3.2-5). During the first 4 d after infection, the wound area increased on average to 110.6% of the original size in the *P. aeruginosa* group and 102.3% of the original size in the *S. aureus* group. After the first debridement, the wound area began to decrease, paralleling a decrease in the microbial load. There was a statistically significant difference ($P < 0.01$) in wound area between the negative control and both infected groups on days 8 and 9; on day 11, only the difference between the negative control and *P. aeruginosa* groups remained significant. Microbial load was similar between the 2 infected groups (5.66 compared with 5.54 log[CFU/swab]) on day 4 and did not differ significantly on days 5, 8, and 11.

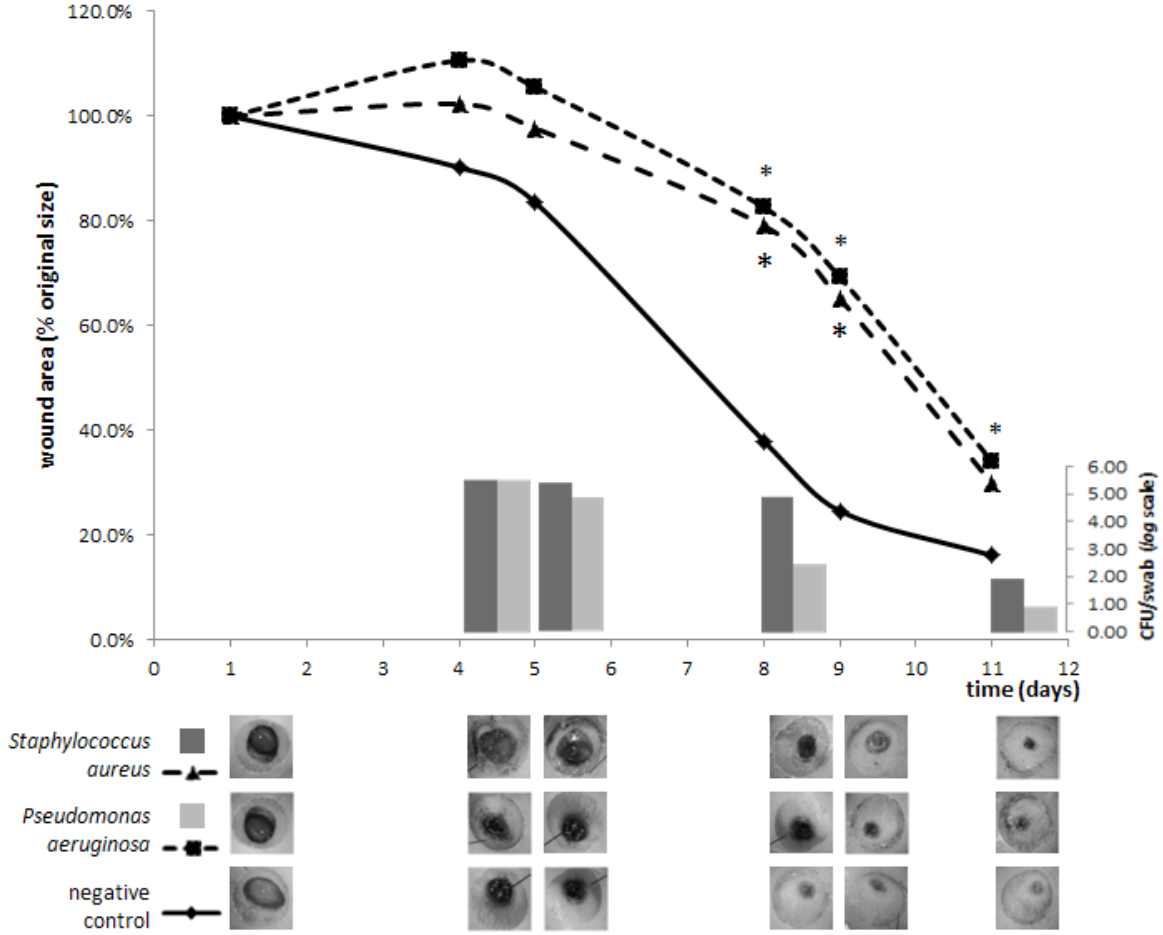


Figure B3.2-5 – Results of the final wound-infection model study. The line graph illustrates wound healing kinetics of the infected (*S. aureus* or *P. aeruginosa*) and negative control groups. Each point represents the mean of the percentage in area of the original wound size. After day 4, wound area decreased progressively and was enhanced after mechanical debridement. Wound area differed significantly (*, $P < 0.01$) between the negative control and both infected groups on days 8 and 9 and between the negative control and *P. aeruginosa*-inoculated groups on day 11. The bar graph illustrate microbial load (from quantitative swab-sample cultures and expressed in no. of cfu per swab [log scale]) between the 2 infected groups on days 4, 5, 8, and 11. The *S. aureus*-inoculated group showed a trend ($0.05 < P < 0.10$) toward increased microbial load on days 5, 8, and 11. The lower panel contains macrophotographs of wounds from a representative rat in each group at each time point.

Histologic analysis

Both EG and DG decreased ($P < 0.01$) as the GT area increased in all groups from day 9 to day 11 (Figure B3.2-6). On day 9, EG closure was significantly delayed in infected groups compared with controls (*S. aureus*, 0.12 ± 0.06 mm; *P. aeruginosa*, 1.3 ± 0.15 mm; control, 1.55 ± 0.15 mm; $P < 0.01$). By day 11, all wounds were completely reepithelialized, except for a single ulcer in the rats inoculated with *P aeruginosa*. GT area did not differ between groups on either day 9 or 11. However, DG closure followed wound contraction, as evaluated by digital planimetry. Wounds of infected rats showed significantly less DG closure than did those of control rats on days 9 (*S. aureus*, 1.88 ± 0.09 mm; *P. aeruginosa*, 2.93 ± 0.08 mm; control, 3.17 ± 0.15 mm; $P < 0.01$) and 11 (*S. aureus*, 1.30 ± 0.16 mm; *P. aeruginosa*, 2.20 ± 0.15 mm; control, 2.43 ± 0.06 mm; $P < 0.01$). DG closure did not differ significantly between infected groups.

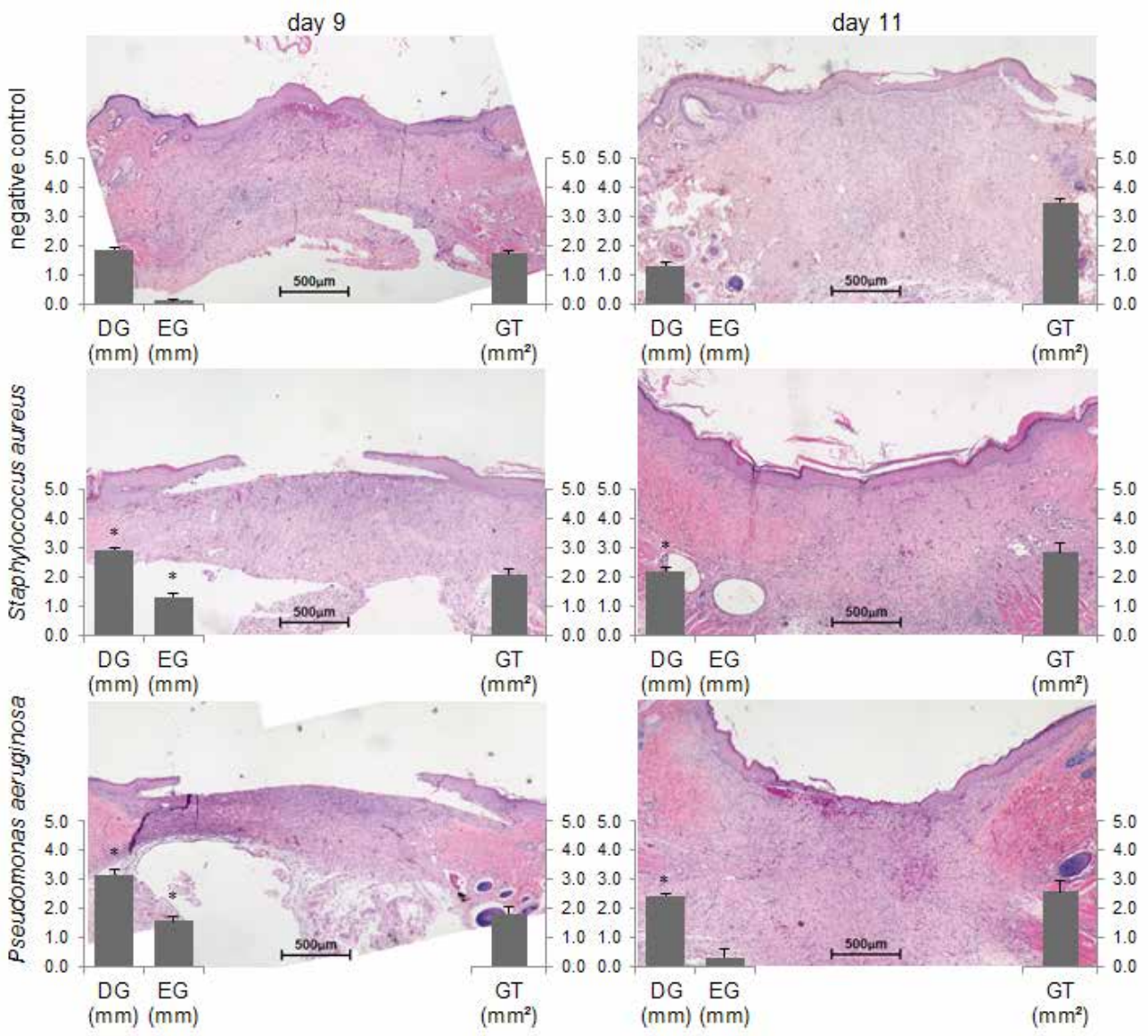


Figure B3.2-6 – Quantitative histologic evaluation of epithelial gap (EG), dermal gap (DG), and granulation tissue (GT) area in the negative control and both infected (*S. aureus* or *P. aeruginosa*) groups on days 9 and 11. On day 9, EG closure in both infected groups was significantly (*, $P < 0.01$) delayed compared with that in the control group. On days 9 and 11, wounds in the infected groups showed significantly (*, $P < 0.01$) less DG closure than did the control group. Original magnification, ×50.

Discussion

The impairment of wound healing by DM, which is potentiated by infection, causes a significant amount of human morbidity and mortality worldwide (5, 29) and has prompted the investigation of new TAT approaches. Although swine are thought to be the ideal large animal model for cutaneous disease (30) the use of rodents overcomes several of its disadvantages (for example, size, cost, housing, husbandry, and reagent validation). Therefore, rodents are still the model of choice for research purposes in this area. However, there are no standardized wound-infection models for testing TAT.

One well-known rodent model (17) is a splinted, excisional wound-healing model that involves genetically diabetic mice (db/db mice). In contrast, we used male Wistar rats in which DM had been chemically induced as a rodent model. Rats are a widely used biomedical research animal and currently the primary model for many preclinical tests, including those in the DM field (31). Although the use of rat models has declined in the last decade, mainly due to broad advances in the development of mouse genetic technologies, rats still afford many advantages over mice, primarily in regard to size and behavioral characteristics (32). In our case, the adhesive-tape jacket needed to prevent dressing loss and subsequent unintentional critical colonization would be impossible to adapt to a smaller rodent. Moreover, we expected our studies to involve frequent, nonpainful procedures. Rats often are easier to train and handle than are mice (33), obviating the need for inhalant anesthesia before procedures and reducing the associated time, distress, and mortality risks. Except for during the initial epilation and wounding procedures, the rats in our current study were restrained simply by placing a surgical drape over the rat's head to maintain immobilization in the absence of any identifiable behavioral stress signs.

We have used streptozotocin-induced diabetic Wistar rats rather than one of the available rat genetic models. Although genetic models offer some undisputed advantages, they are of limited availability and are expensive for regular screening studies of wound healing related to DM (34). Further, the most common and readily available of genetically diabetic rats are obese to the point that excisional wound closure is impeded not only by physiologic dysfunction but also by excess subcutaneous fat (35).

Streptozotocin targets and inhibits pancreatic β-cell function without affecting the exocrine function and produces a DM type 1 phenotype with residual insulin secretion that enables animals to live longer than do genetic models before insulin treatment is needed. However, in addition to this biochemical deficit, this chemical agent causes other effects, such as altered T cell function and decreased macrophage phagocytosis, which also may contribute to impaired healing (9, 36, 37). Nevertheless, the streptozotocin model is one of the most suitable for use in wound healing studies, allowing accurate quantification of the main aspects of a healing wound such as wound closure, reepithelialization, and GT formation. In addition, we used male rats, whose pancreatic islet β cells are more prone to streptozotocin-induced cytotoxicity than are those of female rats (23) and which have 40% stronger skin than do female rats, due to a much thicker dermis (16). Our DM-induction rate exceeded 80%, as expected for the protocol (23), and the postinduction mortality rate was low (less than 5%).

The silicone splint was used in previous models because it minimized wound contraction without affecting the rate of reepithelialization, thus better recapitulating the repair mechanisms underlying human wound healing (16). In our current study, the splint had the additional benefit of creating a sealed artificial chamber over the wound, maintaining sterility at the wound site and allowing the establishment of a monoculture infection while enabling the application of

products in a liquid vehicle. We filled this chamber twice daily with sterile saline, which provided a wet wound microenvironment beneficial to wound healing (38). Sterile saline is also the most frequently used negative TAT control. The influence of the splint and dressing maintenance on wound closure has been reported as a problem (9, 17). When the dressing is breached, the wound becomes contaminated, and splint loss is easier. Once animals lose the splint, wound closure occurs more rapidly. All of these factors can lead to the exclusion of animals from the study; therefore, meticulous application and maintenance of the dressing that protects the splint is an essential aspect of model standardization. To prevent dressing loss, we introduced a jacket made from adhesive tape. With twice-daily evaluation for the integrity of the adhesive-tape jacket and the addition of reinforcement whenever deemed necessary, we had a 100% success rate of maintaining the dressing and splint to the end of the experiment. The jacket seemed to be well tolerated by the rats, which exhibited no overt signs of distress.

Rodent skin is covered with dense hair that undergoes a defined cycle of hair growth similar to that of human hair (35). Although hair removal protocols are described in detail only infrequently in studies, hair regrowth has been reported as an impediment to splint and dressing skin adherence, resulting in the failure of skin contraction restriction and consequent exclusion of animals from the study (39). This problem is particularly important in wound infection models, because razor shaving produces microscopic cuts in the epidermis, and hair growth around the wound site acts as a wick, both of which increase the risk of wound contamination. Cold waxing, as indicated by the hair-density index we used here, was more effective than were shaving or chemical depilatories because waxing removed the hair from beneath the skin surface (40). This improvement in limiting hair growth was made at the expense of persistent skin lesions. However, although cold wax tended to cause more immediate (day 1) epithelial injury, as measured by the skin-damage index, than did depilatory cream, this effect was completely reversed by day 4. This outcome is consistent with previous skin electrical potential studies (40), which showed that waxing causes stratum corneum damage with an immediate loss of cutaneous barrier function and has a rapid and apparently complete recovery by the fifth day after treatment. From our optimization study and based on the previously cited skin electrical potential study (40), we decided to use cold waxing epilation in our final study at 4 d before wounding. This strategy prevented regrowth of hair throughout the course of the experiment and maintained the barrier function of the healthy surrounding skin.

The conversion of an excisional wound model to a wound-infection model is simple enough. The addition of a known concentration of virulent bacteria to the wound bed establishes infection in the test group (30), but the most difficult problem to solve is the prevention of unintentional critical colonization or infection. Although sterilization of the wound is impossible, the use of strict measures can prevent colonization by pathogenic or normal skin flora microorganisms beyond a certain threshold, above which there may be impairment to wound healing. We and others (26, 41) consider this limit to be a tissue microbial load of 10^5 cfu/g tissue or 10^3 cfu/swab. A bioburden below this threshold does not seem to impair tissue repair and frequently has a positive effect on wound healing (42). One potential criticism of our current study is that we did not include a chlorhexidine-based antiseptic solution test group; this type of antiseptic is considered to be ideal for wound infection prevention because of its persistent activity that prevents regrowth of microorganisms for at least 24 h (43). This characteristic was the reason we did not include this type of disinfectant in our model: we would have had to pretreat the infection test group with the antiseptic solution, and its residual antiseptic activity might have limited the development of infection. We chose to use 10% povidone-iodine and 70% isopropyl alcohol, because these agents have an immediate bactericidal action but

minimal residual activity (44). This strategy has been adopted in another animal model of diabetic wound infection (45).

One of the greatest controversies in wound management is the usefulness of quantitative swab cultures for predicting the presence of wound infection. This method has received criticism because it is thought to estimate wound-surface microbial numbers only and not deep-tissue numbers (41). However, several studies in humans and animals have demonstrated high sensitivity (93.5% to 100%) and good specificity (76.3% to 94.2%) and accuracy (approximately 90% to 99%) when compared with tissue-biopsy quantitative cultures (24, 46). In an experimental acute-wound rat model, a tissue count of 10^5 cfu/g was equivalent to a count of 10^3 cfu/mL obtained from a moist swab (47); another study (24) demonstrated that compared with biopsy cultures, one-point quantitative swab cultures detected similar types of microorganisms but underestimated bacterial numbers by a factor of 2 logs. These studies suggest that the quantitative swab method of collection and culturing is acceptable, given its correlation with the invasive method. The invasive method was strongly contraindicated in the model we developed because our study required serial evaluations of the bioburden, along with planimetry by digital photography and a final histologic evaluation. Because these measures constituted the final endpoints and would have been influenced by the wound-bed trauma induced by serial biopsies, we opted for swabbing.

Many methods (48) have been described for swab collection (10-point diagonal method, 1-cm^2-area sampling method, and one-point rotation method), but none has gained universal acceptance. In the current study and in accordance with others (24), we selected the one-point method because of the relatively small size of the wounds and to better avoid contamination with periwound flora. In a murine DM wound-healing model using quantitative cultures and transmission electron microscopy studies (19), the majority of bacteria were in the scab above the wound bed rather than in the wound tissue. In addition, ulcer debridement (that is, the removal of the scab), which is essential to the model we developed, reproduced the current clinical practice of wound debridement previous to microbiologic sampling (41) and theoretically provided an improved estimate of deep-tissue bacterial numbers. Although the numbers of colonies obtained from swab and tissue samples varied, both samples were correlated logarithmically for each bacterium. Our data indicated that the one-point swab culture yielded an average 1.9 log(cfu) under-estimation of colony counts compared with those of the tissue cultures, consistent with the results of several other clinical and experimental studies (24, 47, 49). Surprisingly, we found that when *P. aeruginosa* and *S. aureus* groups were pooled before evaluation, this correlation could not be reestablished. Our results, which show underestimation of *P. aeruginosa* swab samples relative to *S. aureus* swab samples, may be explained by differential distribution of the 2 bacteria in the wound. This notion is in line with a previous study (50), in which confocal laser scanning microscopy of clinical wound-biopsy specimens demonstrated that the distance from *P. aeruginosa* aggregates to the wound surface was greater than that of *S. aureus* aggregates, leading to underestimation of *P. aeruginosa* in swab samples. This result supports the possibility that factors intrinsic to each pathogenic bacteria contribute, as does sampling technique, to the differences reported in studies comparing swab and tissue-sample quantitative cultures. Finally, the choice of selective media (cetrimide or Chapman mannitol salt agar) is important, because using the proper medium is essential for the correct evaluation of target pathogens (41) and to prevent concurrent colonization by other microorganisms. The noninvasiveness of the quantitative swab, which allowed concomitant debridement and revealed a strong correlation with tissue sampling, made this method ideal for use in our rodent wound-infection model.

After optimizing each step of the process, we designed a protocol allowing the simultaneous and serial assessment of microbial load (by using quantitative swab-sample cultures) and the kinetics of wound closure (through digital photography and computerized planimetry software) and a well-defined and easily repeatable quantitative histologic evaluation. It was our requirement that the model not only accurately paralleled the healing of infected wounds in humans but also the current treatment standard of care. Sharp debridement is standard procedure in wound management, and there is no clinical or experimental rationale for using TAT products in its absence (8). Debridement converts the molecular and cellular environment of a chronic wound to that of an acute, healing wound through the removal of scabs and debris. Many studies (7, 19, 51) indicate that debridement plays an important role in removing bioburden and enhancing cicatrization, and its frequency is directly related to the rate of healing (52). Furthermore and most importantly, debridement has been shown in several models to open a time-dependent window for TAT use (7). Debridement led to a significant decrease in the resistance of the bioburden to TAT for as long as 24 h, with resistance increasing to reach the original resistance levels at 72 h.

Our current study using a rat model replicates the current debridement protocol for infected diabetic ulcers (53). Initial debridement (day 4) removed a cellular burden of dead and senescent cells and excessive bacterial load; additional maintenance debridement (days 5 and 8) maintained the wound environment and the readiness of the wound bed for healing. The effects of debridement are manifest in our model: first, by limiting the increase in the size of the wound area after day 4, and second, by accelerating wound-healing kinetics as revealed by the inflections in the infected-wound healing curve, which becomes parallel to the control curve from the third debridement onwards. In the current study, macroscopic wound closure showed a similar trend when compared with that of the EG and DG measured in histologic specimens among the control and infected groups. There was not, however, a statistically significant difference in GT area among groups. This result is probably related to an increase in collagenolytic activity in the wound, generating increased feedback synthesis (54). Qualitative differences in GT between groups may exist, and additional studies, such as complementary breaking-strength measurements, would be of interest. From the analysis of the planimetry and histologic data, we can conclude that in our model, the best time to evaluate differences between the infected and control groups is day 9, and TAT studies probably will not benefit from a longer period of evaluation. In addition, DG closure is a better parameter than is GT area for evaluating the histologic level of contraction of the wound.

In summary, we optimized a new wound infection model in chemically induced diabetic Wistar rats. This model can be used to investigate new approaches to TAT. The model has numerous benefits: the necessary materials and techniques are simple, reproducible, and practical for experiments with large sample sizes. Furthermore, in light of the analogies to human-infected wound healing and treatment, this model can serve as a valid alternative for applied research.

References

1. Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. JAMA. 2005 Jan 12;293(2):217-28.
2. Prompers L, Huijberts M, Apelqvist J, Jude E, Piaggesi A, Bakker K, et al. Optimal organization of health care in diabetic foot disease: introduction to the Eurodiale study. Int J Low Extrem Wounds. 2007 Mar;6(1):11-7.
3. Braiman-Wiksman L, Solomonik I, Spira R, Tennenbaum T. Novel insights into wound healing sequence of events. Toxicol Pathol. 2007;35(6):767-79.
4. Falagas ME, Koletsi PK, Bliziotis IA. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) Acinetobacter baumannii and Pseudomonas aeruginosa. J Med Microbiol. 2006 Dec;55(Pt 12):1619-29.
5. Falanga V. Wound healing and its impairment in the diabetic foot. Lancet. 2005 Nov 12;366(9498):1736-43.
6. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. Clin Infect Dis. 2004 Oct 1;39(7):885-910.
7. Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, et al. Biofilm maturity studies indicate sharp debridement opens a time- dependent therapeutic window. J Wound Care. 2010 Aug;19(8):320-8.
8. Lipsky BA, Hoey C. Topical antimicrobial therapy for treating chronic wounds. Clin Infect Dis. 2009 Nov 15;49(10):1541-9.
9. Scherer SS, Pietramaggiori G, Mathews JC, Chan R, Fiorina P, Orgrill DP. Wound healing kinetics of the genetically diabetic mouse. Wounds. 2008;20(1):18-28.
10. Velander P, Theopold C, Hirsch T, Bleiziffer O, Zuhaili B, Fossum M, et al. Impaired wound healing in an acute diabetic pig model and the effects of local hyperglycemia. Wound Repair Regen. 2008 Mar-Apr;16(2):288-93.
11. Ahn ST, Mustoe TA. Effects of ischemia on ulcer wound healing: a new model in the rabbit ear. Ann Plast Surg. 1990 Jan;24(1):17-23.
12. Romana-Souza B, Nascimento AP, Monte-Alto-Costa A. Propranolol improves cutaneous wound healing in streptozotocin-induced diabetic rats. Eur J Pharmacol. 2009 Jun 2;611(1-3):77-84.
13. Toker S, Gulcan E, Cayc MK, Olgun EG, Erbilen E, Ozay Y. Topical atorvastatin in the treatment of diabetic wounds. Am J Med Sci. 2009 Sep;338(3):201-4.
14. El-Aziz A, El-Banna T, Abo-Kamar A, Ghazal A, AboZahra R. In vitro and in vivo activity of some antibiotics against staphylococcal biofilm and planktonic cells isolated from diabetic foot infections. Journal of American Science. 2010;6(12):760-70.
15. Tsuboi R, Rifkin DB. Recombinant basic fibroblast growth factor stimulates wound healing in healing-impaired db/db mice. J Exp Med. 1990 Jul 1;172(1):245-51.
16. Wong VW, Sorkin M, Glotzbach JP, Longaker MT, Gurtner GC. Surgical approaches to create murine models of human wound healing. J Biomed Biotechnol. 2011;2011:969618.
17. Galiano RD, Michaels Jt, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen. 2004 Jul-Aug;12(4):485-92.
18. Shi CM, Nakao H, Yamazaki M, Tsuboi R, Ogawa H. Mixture of sugar and povidone-iodine stimulates healing of MRSA-infected skin ulcers on db/db mice. Arch Dermatol Res. 2007 Nov;299(9):449-56.

19. Zhao G, Hochwalt PC, Usui ML, Underwood RA, Singh PK, James GA, et al. Delayed wound healing in diabetic (db/db) mice with Pseudomonas aeruginosa biofilm challenge: a model for the study of chronic wounds. Wound Repair Regen. 2010 Sep-Oct;18(5):467-77.
20. Council of the European Communities. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Off J Eur Communities.L358:1-28.
21. Portuguese Agricultural Ministry. Portaria no. 1005/92 of 23 October on the protection of animals used for experimental and other scientific purposes. Diário da República I - Série B.245:4930-42.
22. Institute for Laboratory Animal Research. Guide for the care and use of laboratory animals. 8th ed. Washington DC, USA: National Academies Press; 2011.
23. Wu KK, Huan Y. Streptozotocin-induced diabetic models in mice and rats. Curr Protoc Pharmacol. 2008 Mar;Chapter 5:Unit 5 47.
24. Sullivan PK, Conner-Kerr TA, Hamilton H, Parrish-Smith E, Tefertiller C, Webb A. Assessment of wound bioburden development in a rat acute wound model - quantitative swab versus tissue biopsy. Wounds. 2004;16(4):115-23.
25. Weinstein MP. Diagnostic Technologies in Clinical Microbiology. In: Murray PR, Baron EJ, Jorgensen B, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology. 8th ed. Washington DC, USA: ASM Press; 2003. p. 183-1213.
26. Robson MC, Lea CE, Dalton JB, Heggers JP. Quantitative bacteriology and delayed wound closure. Surg Forum. 1968;19:501-2.
27. Chapman GH. A single culture medium for selective isolation of plasma coagulating staphylococci and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation and the Stone reaction. J Bacteriol. 1946;51:409-10.
28. Brown VI, Lowbury EJ. Use of an improved cetrimide agar medium and other culture methods for Pseudomonas aeruginosa. J Clin Pathol. 1965 Nov;18(6):752-6.
29. Pecoraro RE, Reiber GE, Burgess EM. Pathways to diabetic limb amputation. Basis for prevention. Diabetes Care. 1990 May;13(5):513-21.
30. Greenhalgh DG. Models of wound healing. J Burn Care Rehabil. 2005 Jul-Aug;26(4):293-305.
31. Lee JH, Yang SH, Oh JM, Lee MG. Pharmacokinetics of drugs in rats with diabetes mellitus induced by alloxan or streptozocin: comparison with those in patients with type I diabetes mellitus. J Pharm Pharmacol. 2010 Jan;62(1):1-23.
32. Huang G, Tong C, Kumbhani DS, Ashton C, Yan H, Ying QL. Beyond knockout rats: new insights into finer genome manipulation in rats. Cell Cycle. 2011 Apr 1;10(7):1059-66.
33. Rodier PM. Critical periods for behavioral anomalies in mice. Environ Health Perspect. 1976 Dec;18:79-83.
34. Srinivasan K, Ramarao P. Animal models in type 2 diabetes research: an overview. Indian J Med Res. 2007 Mar;125(3):451-72.
35. Davidson JM. Animal models for wound repair. Arch Dermatol Res. 1998 Jul;290 Suppl:S1-11.
36. Goodson WH, 3rd, Hung TK. Studies of wound healing in experimental diabetes mellitus. J Surg Res. 1977 Mar;22(3):221-7.
37. Seifter E, Rettura G, Padawer J, Stratford F, Kambosos D, Levenson SM. Impaired wound healing in streptozotocin diabetes. Prevention by supplemental vitamin A. Ann Surg. 1981 Jul;194(1):42-50.

38. Vogt PM, Andree C, Breuing K, Liu PY, Slama J, Helo G, et al. Dry, moist, and wet skin wound repair. Ann Plast Surg. 1995 May;34(5):493-9; discussion 9-500.
39. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells. 2007 Oct;25(10):2648-59.
40. Ando HY, Escobar A, Schnaare RL, Sugita ET. Skin potential changes in the guinea pig due to depilation and the repeated application of polyethlyene glycol and retinoic acid. J Soc of Cosmet Chem. 1983;34(3):159-69.
41. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001 Apr;14(2):244-69.
42. Levenson SM, Kan-Gruber D, Gruber C, Molnar J, Seifter E. Wound healing accelerated by Staphylococcus aureus. Arch Surg. 1983 Mar;118(3):310-20.
43. Mayhall CG. Hospital epidemiology and infection control. 3rd ed. Philadelphia, USA: Lippincott Williams & Wilkins; 2004.
44. Maki DG, Ringer M, Alvarado CJ. Prospective randomised trial of povidone-iodine, alcohol, and chlorhexidine for prevention of infection associated with central venous and arterial catheters. Lancet. 1991 Aug 10;338(8763):339-43.
45. Hirsch T, Spielmann M, Zuhaili B, Koehler T, Fossum M, Steinau HU, et al. Enhanced susceptibility to infections in a diabetic wound healing model. BMC Surg. 2008;8:5.
46. Stotts NA. Determination of bacterial burden in wounds. Adv Wound Care. 1995 Jul-Aug;8(4):suppl 46-52.
47. Bornside GH, Bornside BB. Comparison between moist swab and tissue biopsy methods for quantitation of bacteria in experimental incisional wounds. J Trauma. 1979 Feb;19(2):103-5.
48. Bonham PA. Swab cultures for diagnosing wound infections: a literature review and clinical guideline. J Wound Ostomy Continence Nurs. 2009 Jul-Aug;36(4):389-95.
49. Neil JA, Munro CL. A comparison of two culturing methods for chronic wounds. Ostomy Wound Manage. 1997 Apr;43(3):20-2, 4, 6 passim.
50. Fazli M, Bjarnsholt T, Kirketerp-Moller K, Jorgensen B, Andersen AS, Krogfelt KA, et al. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol. 2009 Dec;47(12):4084-9.
51. Schultz GS, Sibbald RG, Falanga V, Ayello EA, Dowsett C, Harding K, et al. Wound bed preparation: a systematic approach to wound management. Wound Repair Regen. 2003 Mar;11 Suppl 1:S1-28.
52. Steed DL, Donohoe D, Webster MW, Lindsley L. Effect of extensive debridement and treatment on the healing of diabetic foot ulcers. Diabetic Ulcer Study Group. J Am Coll Surg. 1996 Jul;183(1):61-4.
53. Steed DL, Attinger C, Colaizzi T, Crossland M, Franz M, Harkless L, et al. Guidelines for the treatment of diabetic ulcers. Wound Repair Regen. 2006 Nov-Dec;14(6):680-92.
54. Bucknall TE. The effect of local infection upon wound healing: an experimental study. Br J Surg. 1980 Dec;67(12):851-5.

Chapter 4: In vivo topical bacteriophage therapy of infected diabetic wounds

Specific framework

The use of animal models is a fundamental part of pharmaceutical research and development in the field of chronic wound infections. In the case of BT, after the pioneering work of Smith et al. (1-4), many reported the examination of bacteriophage preparations efficacy against experimental infections by *S. aureus* (5, 6), *P. aeruginosa* (5, 7), and *A. baumannii* (5), as well as other infectious agents in various animal models. Although the potential of BT for the treatment of infections was well established in these animal models, the reported outcomes varied dramatically, depending on the infectious agents, the animal model of infection, and the lytic potency of the bacteriophages used. Thus, the clinical application of BT requires case-by-case in vivo experimental validation. We developed a protocol to investigate in vivo the antimicrobial activity and wound-healing capability of topically delivered bacteriophage solutions against wounds with chronic *S. aureus*, *P. aeruginosa,* and *A. baumannii* infections. For this purpose, we used our previously optimized rodent wound infection model in chemically induced diabetic Wistar rats (Figure B4.1-1), as well as a pig wound infection model in animals with chemically induced DM (8) (Figure B4.1-2), which suited our needs.

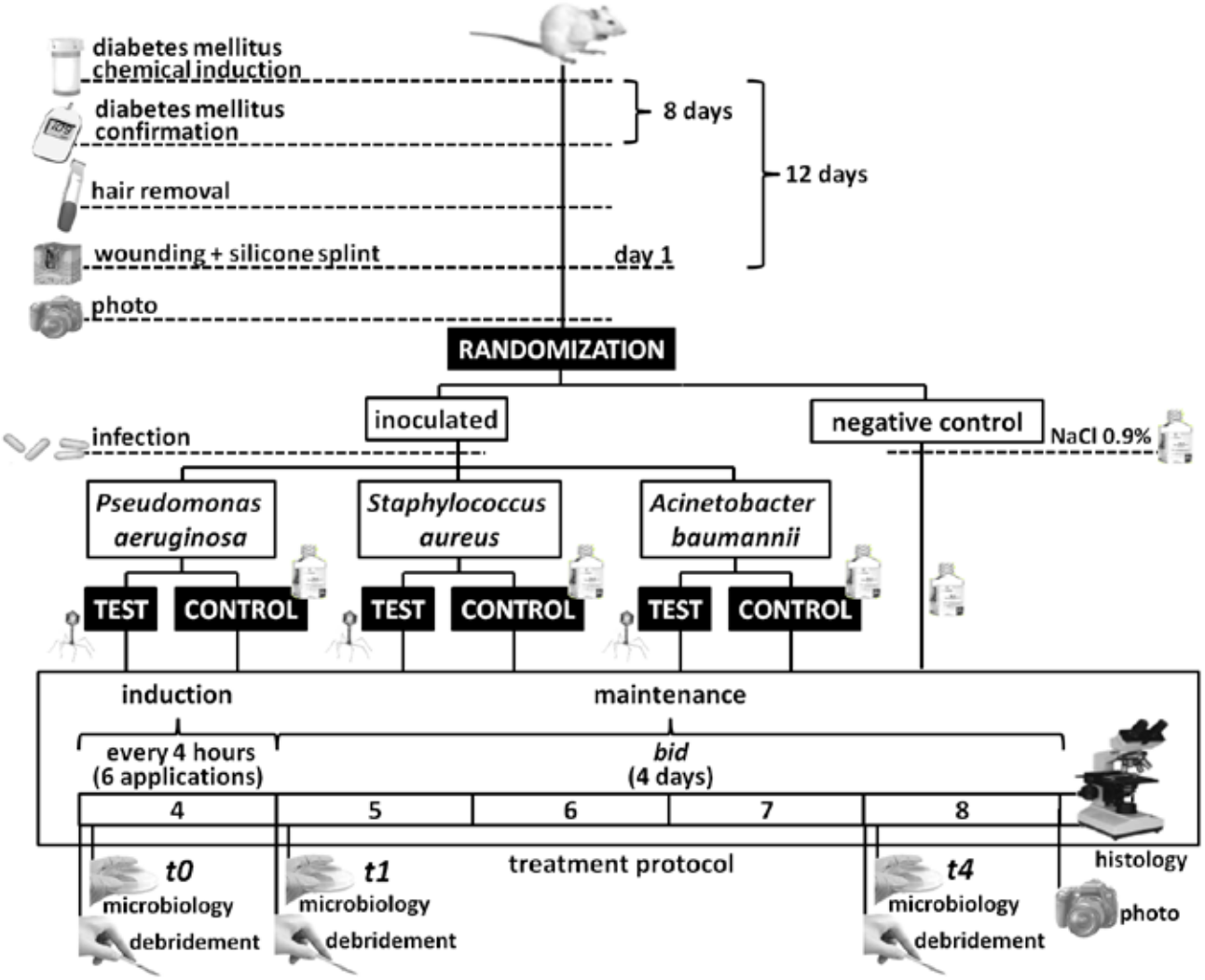


Figure B4.1-1 – Topical bacteriophage therapy protocol in the rodent diabetic chronic wound infection model. The previously optimized rodent wound infection model in chemically induced diabetic Wistar rats was used. Briefly, diabetes mellitus (DM) was chemically induced in specific pathogen-free male Wistar rats. Eight days later, following DM confirmation, 42 diabetic rats were anesthetized. Hair was removed, a full-thickness wound extending through the panniculus carnosus was created in the interscapular region of the upper back using a punch biopsy instrument, and a silicone splint was fixed to the skin. Wounds were photographed using a mounted digital microscope, covered with a semi-occlusive, non-woven polyester dressing, and then maintained in place with a jacket made from adhesive tape. The rats were then randomly divided into 7 groups: negative control (n = 6), *S. aureus*-inoculated control (n = 6) and test (n = 6), *P. aeruginosa*-inoculated control (n = 6) and test (n = 6), and *A. baumannii*-inoculated control (n = 6) and test (n = 6). The wounds of the animals in the negative control group were injected with sterile saline, whereas the wounds of the inoculated groups (test and control) were injected with 2×10^6 cfu of *S. aureus*, *P. aeruginosa,* or *A. baumannii* resuspended in sterile saline. On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off, and the wounds were debrided. All test groups underwent a bacteriophage treatment protocol that consisted of an induction phase and a maintenance phase. The induction phase occurred after the first debridement (post-wounding day 4) and was comprised of six primary bacteriophage cocktail administrations (every 4 hours). The maintenance phase was from day 5 to day 8 and consisted of twice-daily (every 12 hours) primary bacteriophage cocktail administrations. If debridement was performed, bacteriophage administration followed. The control groups received

sterile saline at the same frequency. On days 4, 5, and 8 post-wounding and after debridement, microbial load was evaluated by using the sterile-swab, one-point method. Prior to sacrifice on day 9 post-wounding, the wounds were photographed using a mounted digital microscope for the macroscopic evaluation of wound closure. All animals were sacrificed on day 9 post-wounding, and the ulcers were collected for the histologic evaluation of wound closure.

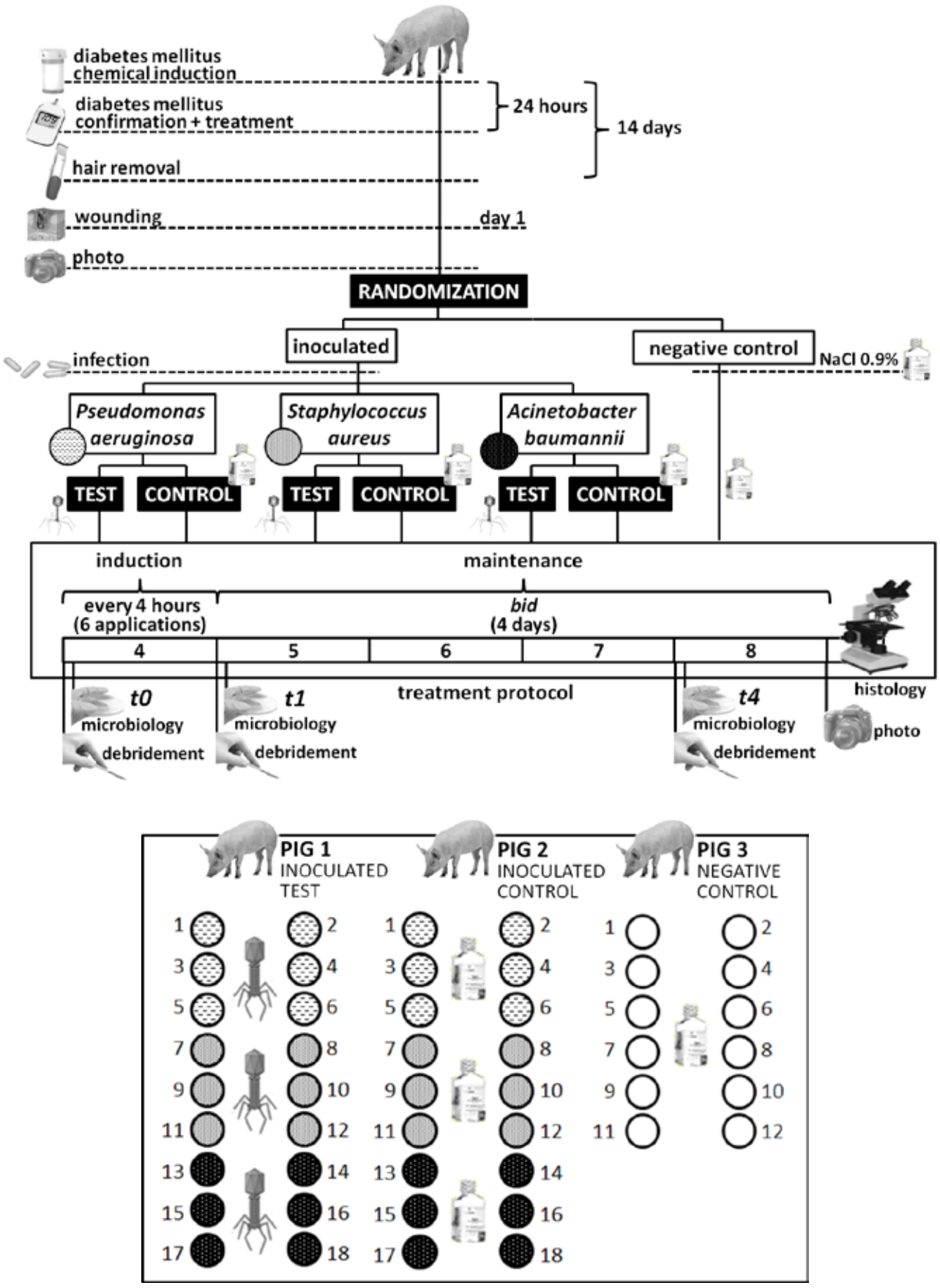


Figure B4.1-2 – Topical bacteriophage therapy protocol in the pig chronic wound infection model. A previously optimized pig wound infection model in animals with chemically induced DM (8) was modified to fit our needs. Three animals (negative control, inoculated-control, and inoculated-test)

with a total of 48 excisional wounds (12 negative control wounds, 12 *S. aureus*-inoculated wounds, 12 *P. aeruginosa*-inoculated wounds, and 12 *A. baumannii*-inoculated wounds) were used in this study. Diabetes mellitus (DM) was chemically induced in 3 female Yorkshire pigs. Blood glucose was measured on a daily basis, and the pigs received daily, subcutaneous insulin injections. Fourteen days after DM induction, the pigs were anesthetized and mechanically ventilated. Hair was removed and full-thickness excisional wounds were created on each side of the paraspinal area (18 in total for each of the inoculated pigs, and 12 in total for the negative control pig) using a punch biopsy instrument. The wounds were photographed using a mounted digital microscope. A modified adhesive chamber made from a colostomy bag was placed over each wound, which was covered with a semi-occlusive, non-woven polyester dressing. In the inoculated animals, wounds were divided into 3 subgroups: *S. aureus* (2 × 6 ulcers); *P. aeruginosa* (2 × 6 ulcers), and *A. baumannii* (2 × 6 ulcers), and inoculated with 2×10^6 cfu of the respective pathogen. Whereas, in the negative control group (12 ulcers), wounds were injected with sterile saline. On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off, and the wounds were debrided. All test groups underwent a bacteriophage treatment protocol that consisted of an induction phase and a maintenance phase. The induction phase occurred after the first debridement (day 4 post-wounding) and was comprised of 6 primary bacteriophage cocktail administrations (every 4 hours). The maintenance phase was from day 5 to day 8 and consisted of twice-daily (every 12 hours) 100 µL primary bacteriophage cocktail administrations. If debridement was performed, bacteriophage administration followed. The control groups received sterile saline with the same frequency. On days 4, 5, and 8 post-wounding and after debridement, microbial load was evaluated by using the sterile-swab, one-point method. In the negative control group, ulcers with more than 10^3 cfu/swab on any given day were considered critically colonized and were excluded from further analysis. Prior to sacrifice on day 9 post-wounding, the wounds were photographed using a mounted digital microscope for the macroscopic evaluation of wound closure. All animals were sacrificed on day 9 post-wounding, and the ulcers were collected for the histologic evaluation of wound closure.

References

1. Smith HW, Huggins MB. Effectiveness of phages in treating experimental Escherichia coli diarrhoea in calves, piglets and lambs. J Gen Microbiol. 1983 Aug;129(8):2659-75.
2. Smith HW, Huggins MB, Shaw KM. The control of experimental Escherichia coli diarrhoea in calves by means of bacteriophages. J Gen Microbiol. 1987 May;133(5):1111-26.
3. Smith HW, Huggins MB, Shaw KM. Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. J Gen Microbiol. 1987 May;133(5):1127-35.
4. Smith HW, Huggins MB. Successful treatment of experimental Escherichia coli infections in mice using phage: its general superiority over antibiotics. J Gen Microbiol. 1982 Feb;128(2):307-18.
5. Soothill JS. Treatment of experimental infections of mice with bacteriophages. J Med Microbiol. 1992 Oct;37(4):258-61.
6. Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. Antimicrob Agents Chemother. 2005 Mar;49(3):1220-1.
7. Soothill JS. Bacteriophage prevents destruction of skin grafts by Pseudomonas aeruginosa. Burns. 1994 Jun;20(3):209-11.
8. Hirsch T, Spielmann M, Zuhaili B, Koehler T, Fossum M, Steinau HU, et al. Enhanced susceptibility to infections in a diabetic wound healing model. BMC Surg. 2008;8:5.

Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds.

Mendes JJ, Leandro C, Corte-Real S, Barbosa R, Cavaco-Silva P, Melo-Cristino J, Górski A, Garcia M.
Wound Repair Regen. 2013 Jul-Aug;21(4):595-603. doi: 10.1111/wrr.12056.
Epub 2013 Jun 11.
PMID: 23755910 [PubMed - indexed for MEDLINE]

Abstract

Chronic wounds that fail to heal are a common complication of diabetes mellitus and the most common precipitating reason for nontraumatic lower limb amputation. Unfortunately, the bacterial species that cause these infections are becoming more resistance to antibiotics, making them increasingly difficult to treat. We assessed the feasibility of combating chronic bacterial infections with a topically delivered bacteriophage cocktail in two animal models of diabetes mellitus. Microbiological, planimetric, and histological parameters were compared in debrided infected wounds with or without topical bacteriophage treatment. We determined that bacteriophage treatment effectively decreased bacterial colony counts and improved wound healing, as indicated by smaller epithelial and dermal gaps, in *S. aureus* and *P. aeruginosa* infections but was not as effective against *A. baumannii*. Although the improvements were more significant in the rodent model than in the porcine model, our results suggest that topically administered bacteriophage treatment may be effective in resolving chronic infections, especially when applied in conjunction with wound debridement. These findings have important implications for the feasibility of using topical antimicrobial therapies to safely treat chronic infections in diabetes mellitus patients.

Introduction

Diabetic foot infections (DFIs) are a frequent and serious complication of diabetes mellitus (DM) and are the world's leading cause of nontraumatic lower limb amputation (1). In current clinical practice, DFI treatment includes debridement and systemic antibiotics (2). However, because of deficient vascularization and insufficient local antibiotic concentrations, these treatments are often ineffective (3). In addition, multi-drug resistant organisms, such as methicillin-resistant *S. aureus* and pan-drug-resistant nonfermenting Gram-negative bacilli, threaten the efficacies of these therapies in both community-dwelling and hospitalized patients (4, 5). Thus, it is necessary to identify new therapeutic strategies for DFIs. Topical treatment is advantageous because it avoids adverse effects from systemic treatment, increases target site concentration, and allows the use of agents that are not suitable for systemic therapy. Mechanical debridement remains pivotal to this strategy because it significantly reduces the bioburden, and as elegantly demonstrated by Wolcott et al. (6), opens a time-dependent therapeutic window for topical antimicrobial therapy (TAT). Nevertheless, no TAT agent has been proven to effectively treat DFI to date (7).

Bacteriophages are small viral entities, existing as nucleic acids packaged within a protein capsid, that specifically infect bacteria. Depending on their nature, after injection of their nucleic acids inside the bacteria, bacteriophages can either reside as a stable element called prophage inside the host cell as a free plasmid molecule or integrated into the host chromosome (temperate bacteriophages), or induce lysis of the bacterial host with the release of newly formed viral particles (lytic bacteriophages) (8). If appropriately complemented by adequate mechanical debridement, lytic bacteriophages could be efficient TAT agents in selected clinical environments because of their specificity and efficiency in lysing pathogenic bacteria, including those associated with multi-drug resistance (9). Moreover, they are not pathogenic in animals or humans (10), effectively eliminate bacteria in biofilms, and are active even in microaerophilic environments with high bacterial loads (11).

Bacteriophage therapy is widely used and generally accepted as safe and beneficial in some parts of the world (12), and recent trials in animal models have demonstrated their potential to improve or heal bacterial skin infections following both internal (13) and external application (14, 15). However, there is little experimental evidence demonstrating that bacteriophages can cure chronic infections established for more than several hours (16).

The aim of this study was to investigate the antimicrobial activity and wound-healing capability of topically delivered bacteriophage solutions against wounds with chronic *S. aureus*, *P. aeruginosa,* and *A. baumannii* infections in two animal models of DM (rat and porcine).

Materials and methods

This study was approved locally by the Animal Ethics Committee of the Instituto de Medicina Molecular and nationally by the Portuguese General Directorate of Veterinary Services (Direcção Geral de Veterinária), in accordance with Portuguese law. All animals were maintained in accordance with European Directive 86/609/EC (17), Portuguese law (Portaria 1005/92) (18), and the *Guide for the Care and Use of Laboratory Animals* (NRC 2011) (19).

Bacterial strains

S. aureus, *P. aeruginosa,* and *A. baumannii* strains were isolated from clinical skin wound samples that were collected from patients and identified in Lisbon-area hospitals. All host strains were stored in tryptone soy broth (Biokar Diagnostics, Pantin Cedex, France) with 15% glycerol (w/v) at -70°C until needed. For all experiments, single colonies were grown overnight on tryptone soy agar (TSA, Biokar

Diagnostics) at 37°C. After 24-h incubation, bacterial cells were suspended in saline (NaCl 0.9%, Applichem, Darmstadt, Germany) and adjusted to McFarland's scale 0.5 (bioMérieux, Craponne, France) with a subsequent 1:10 dilution, producing a final solution concentration of 2.0×10^7 cfu/mL.

Bacteriophages

S. aureus F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 lytic bacteriophages were isolated from sewage water from the Lisbon area. Standard methods (20) for bacteriophage isolation and amplification were employed using the host strains described above. To produce bacteriophage stocks in sufficient quantities for experiments, a previously described protocol of amplification, concentration by high-speed centrifugation, and purification on a cesium chloride gradient (21) was used. Final concentrations were determined with double agar overlay plaque assays (22).

Three primary cocktails (*S. aureus* cocktail, *P. aeruginosa* cocktail, and *A. baumannii* cocktail) and one final cocktail were prepared using different concentrations and relative proportions of purified bacteriophages (Figure B4.2-1).

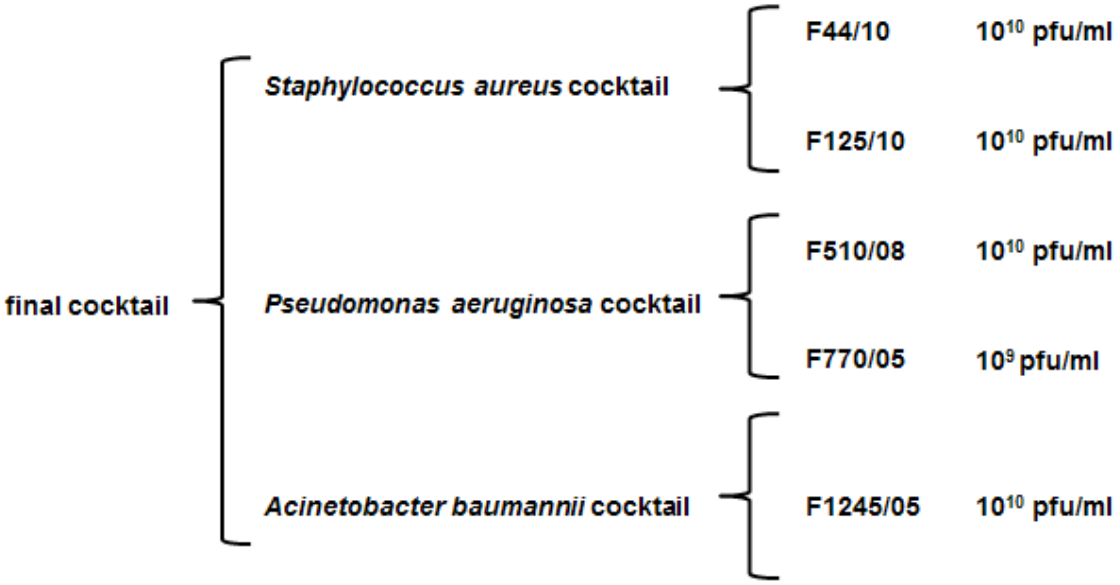


Figure B4.2-1 – Schematic depicting bacteriophage cocktail preparation.

Rodent model

A previously optimized rodent wound infection model in chemically induced diabetic Wistar rats was employed to investigate new approaches to TAT (23). Briefly, specific pathogen-free male Wistar rats [Crl:WI(Han)], weighing 250-350 g (8-10 weeks old) were obtained from Charles River Laboratories (L'Arbresle, Cedex, France). The animals were housed in an approved animal care center, and all surgical procedures were performed in a sanitized surgery room using autoclave-sterilized instruments. DM was chemically induced as described by Wu et al. (24). Eight days later, following DM confirmation, 42 diabetic rats were anesthetized. Their dorsal surface hair was trimmed with an electric clipper, remaining hair removed using cold wax strips. Four days later, the animals were again anesthetized, and a round wound was inflicted by making a 6-mm diameter single full-thickness incision extending through the panniculus carnosus muscle in the interscapular region of the upper back of each rat using a punch biopsy instrument. Immediate-bonding cyanoacrylate glue was used to fix an oval-shaped silicone splint to the skin, and interrupted 3-0 nylon sutures were placed to

maintain its position. Before dressing, wounds were photographed from a standard 1.5-cm distance using a mounted digital microscope (SuperEyes 200× USB Digital Microscope, Shenzhen Tak and Assistive Technology, Shenzhen, China). Wound and surrounding area were covered with a previously tailored, semi-occlusive, non-woven polyester dressing, and maintained in place throughout the entire course of the experiment with a jacket made from adhesive tape.

After applying the dressing but before the animals were conscious, they were randomly divided into seven groups: negative control (n = 6), *S. aureus*-inoculated control (n = 6) and test (n = 6), *P. aeruginosa*-inoculated control (n = 6) and test (n = 6), and *A. baumannii*-inoculated control (n = 6) and test (n = 6). Wounds of the animals in the negative control group were injected with 100 µL sterile saline, whereas wounds of the inoculated groups (test and control) were injected with 100 µL cultured *S. aureus*, *P. aeruginosa,* or *A. baumannii* resuspended in sterile saline by inserting a 27G/19-mm needle attached to a 1-mL disposable syringe through the silicon splint at a 45º angle. On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off, and the wounds were debrided. Debridement consisted of the strict, aseptic, mechanical removal of the scab, defined as a crust of dried blood, serum, and exudate.

All test groups underwent a bacteriophage treatment protocol that consisted of an induction phase and a maintenance phase. The induction phase occurred after the first debridement (post-wounding day 4) and was comprised of six 100-µL primary bacteriophage cocktail administrations (every 4 hours). The maintenance phase was from day 5 to day 8 and consisted of twice-daily (every 12 hours) 100 µL primary bacteriophage cocktail administrations. If debridement was performed, bacteriophage administration followed. The control groups received 100 µL sterile saline with the same frequency.

On days 4, 5, and 8 post-wounding and after debridement, a liquid Amies elution swab (eSwab Collection and Preservation System, Copan, Corona, CA) was used to collect and transport swab cultures. Bacteria collection was performed using the one-point method described by Sullivan et al. (25). Briefly, a sterile swab was used to scrub the center surface of each wound by rotating the swab three times clockwise with enough manual pressure to produce a small amount of exudate. The swab was then inserted into the tube and transported to the laboratory for immediate processing. The swab collection tube was vortexed (with the swab inside) for 5 seconds, and a 100-µL aliquot of the resulting suspension was used for serial dilutions. Quantification was performed using the 10-fold serial dilution method (26). In the inoculated groups, 100 µL of each dilution was plated onto the respective selective media plates: Chapman mannitol salt agar (Biokar Diagnostics) for *S. aureus*, cetrimide agar (Merck Chemical) for *P. aeruginosa* and CHROmagar Acinetobacter for *A. baumannii* (CHROmagar, Paris, France). In the *A. baumannii*-inoculated and negative control groups, 100 µL of each dilution was simultaneously inoculated onto TSA media plates (Biokar Diagnostics). The plates were incubated under aerobic conditions at 37°C for 24 hours, at which time colony counts were performed. The isolates grown on Chapman mannitol salt agar were presumptively identified as *S. aureus* based on colony morphology and mannitol salt agar fermentation (27). The isolates grown on cetrimide agar were presumptively identified as *P. aeruginosa* based on colony morphology (28). The isolates grown on CHROmagar Acinetobacter were identified as *A. baumannii* based on red colony color (29). Prior to sacrifice on post-wounding day 9, wounds were photographed from a standard 1.5-cm distance using a mounted digital microscope as previously described. Wound kinetics were quantified using image-processing software (ImageJ, National Institutes of Health, Bethesda, MD) to measure the wound area by planimetry, and wound area was expressed as a percentage of the initial wound area. All animals were sacrificed by an i.p. injection of pentobarbital (200 mg) on day

9 post-wounding, and each ulcer and the surrounding 0.5-cm skin border was harvested with sterile surgical scissors and placed in a tube. The samples were fixed in 10% buffered formalin solution, and after overnight fixation, they were trimmed and cut through at the widest margin, embedded in paraffin, and sectioned in 3-µm increments. Sections were made perpendicular to the anterior-posterior axis and perpendicular to the wound surface. For each wound, two serial sections were placed on a slide and stained with hematoxylin and eosin. The sections were photographed using a motorized inverted bright-field microscope (Zeiss Axiovert200M, Göttingen, Germany) equipped with a color camera (Leica DM2500, Leica Microsystems GmbH, Wetzlar, Germany) at 50× magnification. Panoramic cross-sectional digital images of each wound were prepared with automated microscopy software (MetaMorph, MDS Analytical Technologies, Sunnyvale, CA) and image-processing software (ImageJ). Each image was analyzed for epithelial gap (EG) and dermal gap (DG) using the same image-processing software. EG was defined as the distance between the advancing edges of clear, multi-layered neoepidermis (30, 31), and its size was measured in millimeters; a completely re-epithelialized wound corresponded to an EG score of zero. DG was defined as the distance between uninjured dermis on both sides of the wound (30, 31) and was measured in millimeters. All wound kinetics and histological measurements were done with the investigator blinded as to sample origin (test or control).

Pig model

A previously optimized pig wound infection model in animals with chemically induced DM (32) was modified to fit our needs. Three animals (negative control, inoculated-control, and inoculated-test) with a total of 48 excisional wounds (12 negative control wounds, 12 *S. aureus*-inoculated wounds, 12 *P. aeruginosa*-inoculated wounds, and 12 *A. baumannii*-inoculated wounds) were used in this study.

Three female Yorkshire pigs weighing approximately 60 kg at arrival were allowed to acclimatize for 1 week prior to the experiment. Animals were housed individually in cages, had free access to water, and were fed twice daily with a standard diet. Pigs were kept in a containment device during all procedures. Pigs were fasted for 12 hours before DM induction. On the day of the procedure, the animals were weighed and given intramuscular anesthesia with xylazine hydrochloride and ketamine hydrochloride. While they were under anesthesia, a 21-gauge intravenous (i.v.) catheter was inserted into an ear vein. Streptozotocin (150 mg/kg body weight diluted in 10 mL/g sterile saline and sterilized by filtration) was administered through the catheter over 1 min. After recovering from anesthesia, post-procedural anti-emetic therapy with metoclopramide was administered. Pigs were continuously observed for the first 3 hours, and then food was offered *ad libitum* to prevent hypoglycemia. Blood glucose was measured on a daily basis, and pigs received daily, subcutaneous injections of 16 IU pre-mixed neutral suspension of neutral (30%) and isophane insulin (70%) (Mixtard 30, Novo Nordisk, Bagsværd, Denmark) to keep blood glucose concentrations between 250 and 400 mg/dL. Fourteen days after DM induction, pigs received induction anesthesia as previously described. They underwent endotracheal intubation and were mechanically ventilated with a volume-limited, time-cycled ventilator (Mark 9; Bird Corporation, Palm Springs, CA) on a mixture of room air and titrated isoflurane (0.5% to 1.5%). The tidal volume was set at 12 mL/kg, and the ventilator rate was 12 breaths/minute. Prior to surgery, the dorsal surface hair was trimmed with an electric clipper, the remaining hair was removed with cold wax strips, and the paraspinal area was thoroughly disinfected using 10% povidone-iodine paint and washed with 70% isopropanol 15 minutes later. For the inoculated pigs, nine full-thickness excisional wounds (6-mm diameter, 6-mm depth) were

created on each side of the paraspinal area (18 in total) using a 6-mm diameter biopsy punch. For the negative control pig, only six excisional wounds were created on each side of the paraspinal area (12 in total). Subsequently, sterile forceps and a surgical blade were used to remove the full-thickness skin flap, and sterile gauze was used to remove coagulated blood and control bleeding. The wounds were photographed from a standard height using a mounted digital microscope. Afterwards, a modified adhesive chamber made from a colostomy bag (two-piece 35-mm Ostomy, Hollister Inc., Libertyville, IL) was placed over each wound, covered with a semi-occlusive, non-woven polyester dressing, and secured in place with surgical staples (Manipler AZ, B. Braun, Tuttlingen, Germany) and adhesive bandages. In the inoculated animals, wounds were divided into three subgroups: *S. aureus* (2 × 6 ulcers); *P. aeruginosa* (2 × 6 ulcers), and *A. baumanni* (2 × 6 ulcers). To immerse the enclosed surface, wounds were respectively inoculated with 2×10^6 cfu of *S. aureus, P. aeruginosa, or A. baumanni* in 100 µL total solution (sterile 0.9% saline). In the negative control group (12 ulcers), wounds were injected with 100 µL sterile saline. After recovering from anesthesia, post-procedural anesthesia (buprenorphine 0.005 mg/kg) and anti-emetic therapy was given every 12 hours for 48 hours. On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off, and the wound debrided as described for the rodent model.

A two-part bacteriophage treatment protocol similar to that employed in the rodent model was used. The induction phase began after the first debridement (post-wounding day 4) and consisted of 100 µL final bacteriophage cocktail administrations every 4 hours for 24 hours. The maintenance phase took place between days 5 to 8 and consisted of twice-daily (every 12 hours) 100 µL final bacteriophage cocktail administrations. Bacteriophage administration followed every debridement session. The control groups received 100 µL sterile saline at the same time.

We employed a microbiological analysis protocol similar to the rodent study. On days 4, 5, and 8 post-wounding and after debridement, a liquid Amies elution swab was used to collect and transport swab cultures, which were processed using the same methods described for the rodent study. Bacteria collection was performed using the one-point method described by Sullivan et al. (25). The swab was then inserted into the tube and transported to the laboratory for immediate processing. Quantification was performed using the 10-fold serial dilution method (26). In the inoculated group samples, 100 µL per dilution was plated onto their respective selective media plates: Chapman mannitol salt agar, cetrimide agar, and CHROmagar Acinetobacter. In the negative control group samples, 100 µL of each dilution was inoculated onto TSA media plates. The plates were incubated under aerobic conditions at 37°C for 24 hours, after which colony counts were performed. The isolates were presumptively identified as previously described. In the negative control group, ulcers with more than 10^3 cfu/swab on any given day were considered to be critically colonized and were excluded from further analysis. Wounds were again photographed on post-wounding day. Wound kinetics were quantified using image-processing software as described above. Wound area was expressed as a percentage of the initial wound area. All animals were sacrificed by i.v. injection of pentobarbital on day 9 post-wounding, and each ulcer (including a 0.5-cm skin border) was entirely harvested using sterile surgical scissors and placed in a tube. For histological studies, the samples were processed and photographed, and the images were analyzed for epithelial gap (EG) using the same methods as described above.

Statistical analysis

All quantitative microbiological results are presented as the mean ± standard deviation and expressed as logarithm-transformed values [log(cfu/swab) for swab samples and log(cfu/ulcer) for

tissue samples]. The data were compared using a logarithmic scale because of wide variations in cfu/swab among samples. Planimetric and histological results are expressed as the mean ± standard deviation. For all datasets, comparisons between groups were performed using two-tailed Student t-tests, and P values < 0.05 were considered significant. All data was entered into a spreadsheet program (Excel, Microsoft, Redmond, WA) for statistical analysis. Analytical statistics were performed by Analyse-it, version 2.21 Excel 12+ (Analyse-it Software, Leeds, UK), a statistical add-in program for Excel.

Results

Rodent model

The results of the microbiological study are presented in Figure B4.2-2 A. Before treatment (*t0*), the average swab colony count in selective media for the *S. aureus*-inoculated, *P. aeruginosa*-inoculated, and *A. baumannii*-inoculated groups were 5.62 ± 0.28 log(cfu/swab), 5.55 ± 0.34 log(cfu/swab), and 2.80 ± 0.68 log(cfu/swab), respectively. The average colony count in non-selective media for the *A. baumannii*-inoculated groups was 5.95 ± 0.23 log(cfu/swab). There were no statistically significant differences between the test and control subgroups. After induction therapy (*t1*), there was a statistically significant difference in colony count in selective media between control and test subgroups in *S. aureus*-inoculated [control, 5.43 ± 0.49 log(cfu/swab); test, 2.47 ± 1.41 log(cfu/swab); P < 0.01], *P. aeruginosa*-inoculated [control, 4.91 ± 0.55 log(cfu/swab); test, 0.69 ± 0.67 log(cfu/swab); P < 0.01], and *A. baumannii*-inoculated groups [control, 2.70 ± 0.77 log(cfu/swab); test, 0.79 ± 0.95 log(cfu/swab); P = 0.01]. There was no statistically significant difference in average colony count in non-selective media between the *A. baumannii*-inoculated test and control subgroups. On day 4 after treatment initiation *(t4*), there was a statistically significant difference in colony count in selective media between control and test subgroups for *S. aureus* inoculation [control, 5.02 ± 0.62 log(cfu/swab); test, 2.40 ± 0.50 log(cfu/swab); P < 0.01], *P. aeruginosa* inoculation [control, 2.56 ± 0.94 log(cfu/swab); test, 0.00 log(cfu/swab); P < 0.01], and *A. baumannii* inoculation [control, 3.03 ± 0.45 log (cfu/swab); test, 0.00 log(cfu/swab); P < 0.01]. There was no significant difference between the *A. baumannii*-inoculated test and control subgroups. From *t0* to *t4* in the *S. aureus* and *P. aeruginosa*-inoculated control subgroups, there was a tendency for microbial load reduction, but statistical significance was only obtained in the *P. aeruginosa*-inoculated group (P < 0.01).

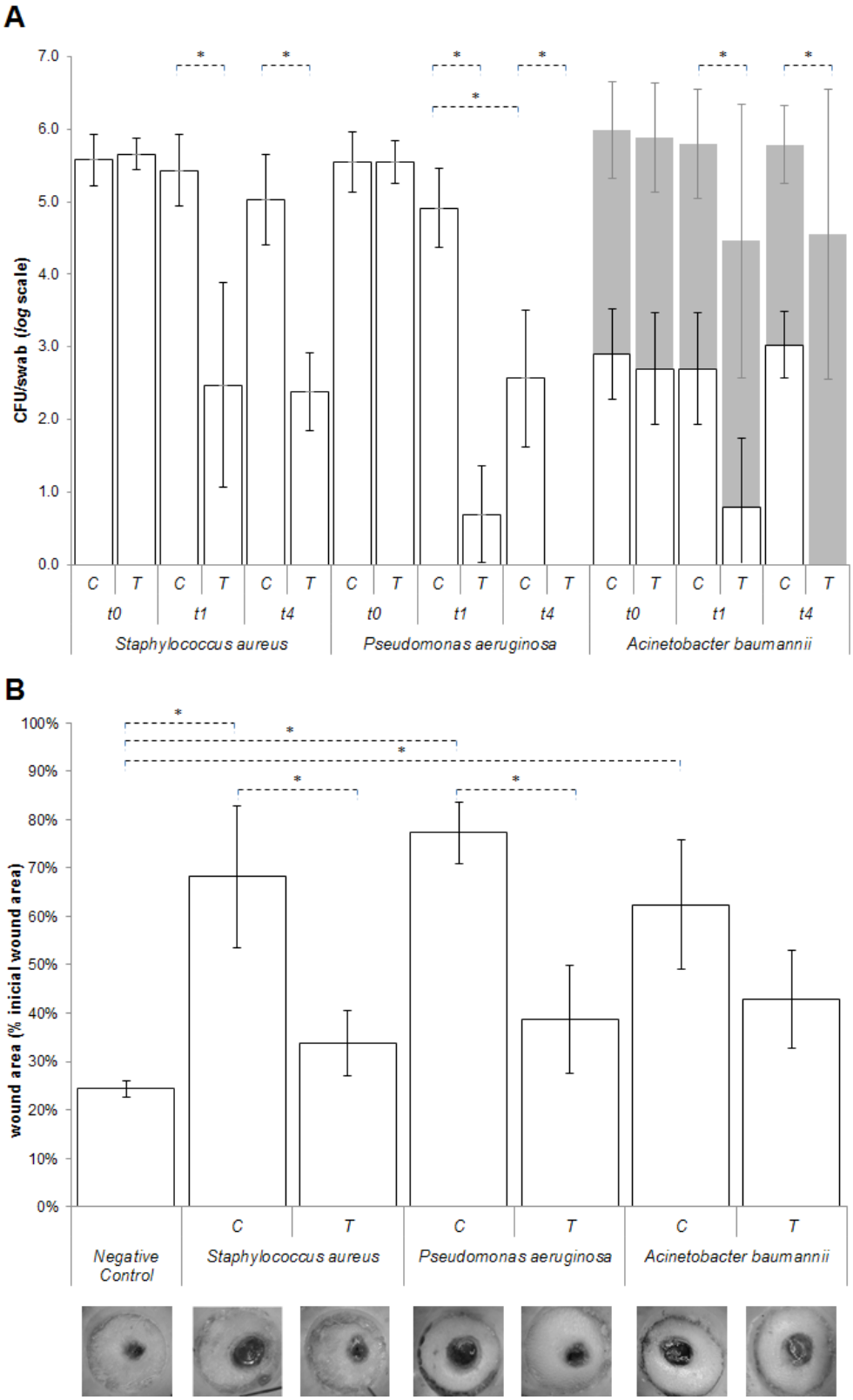


Figure B4.2-2 – (A) Average swab colony count in infected rats. Wounds were swabbed at t0, t1, and t4, and the number of bacterial colony-forming units were compared between control and test conditions for each group. The bacteriophage-treated animals showed significantly lower counts than the control animals in all three groups on t1 and t4. C, control, T, test; White bars, selective media; gray bars, non-selective media; *P < 0.05; (B) Wound closure kinetics in rats. Wound area was assessed on t1 and t9, and the differences between the two timepoints were calculated. Bacteriophage treatment only reduced wound size in *S. aureus*- and *P. aeruginosa*-infected wounds. C, control, T, test; *P < 0.05.

The wound closure kinetics (planimetric) results are presented in Figure B4.2-2 B. There was a statistically significant difference in wound area between the negative control group and all inoculated control subgroups (negative control, 24.3 ± 1.8%; *S. aureus*-inoculated control, 68.2 ± 14.6%, $P < 0.01$; *P. aeruginosa*-inoculated control, 77.3 ± 6.4%, $P < 0.01$; *A. baumannii*-inoculated control, 62.3 ± 13.3%, $P < 0.01$). There was a statistically significant difference between control and test subgroups wound areas in the *S. aureus*-inoculated (test, 33.7 ± 6.7%, $P < 0.01$) and *P. aeruginosa*-inoculated groups (test, 38.7 ± 11.1%, $P < 0.01$). Although there was a tendency for wound area reduction between control and test subgroups, it did not reach statistical significance in the *A. baumannii*-inoculated group (test, 42.9 ± 10.2%).

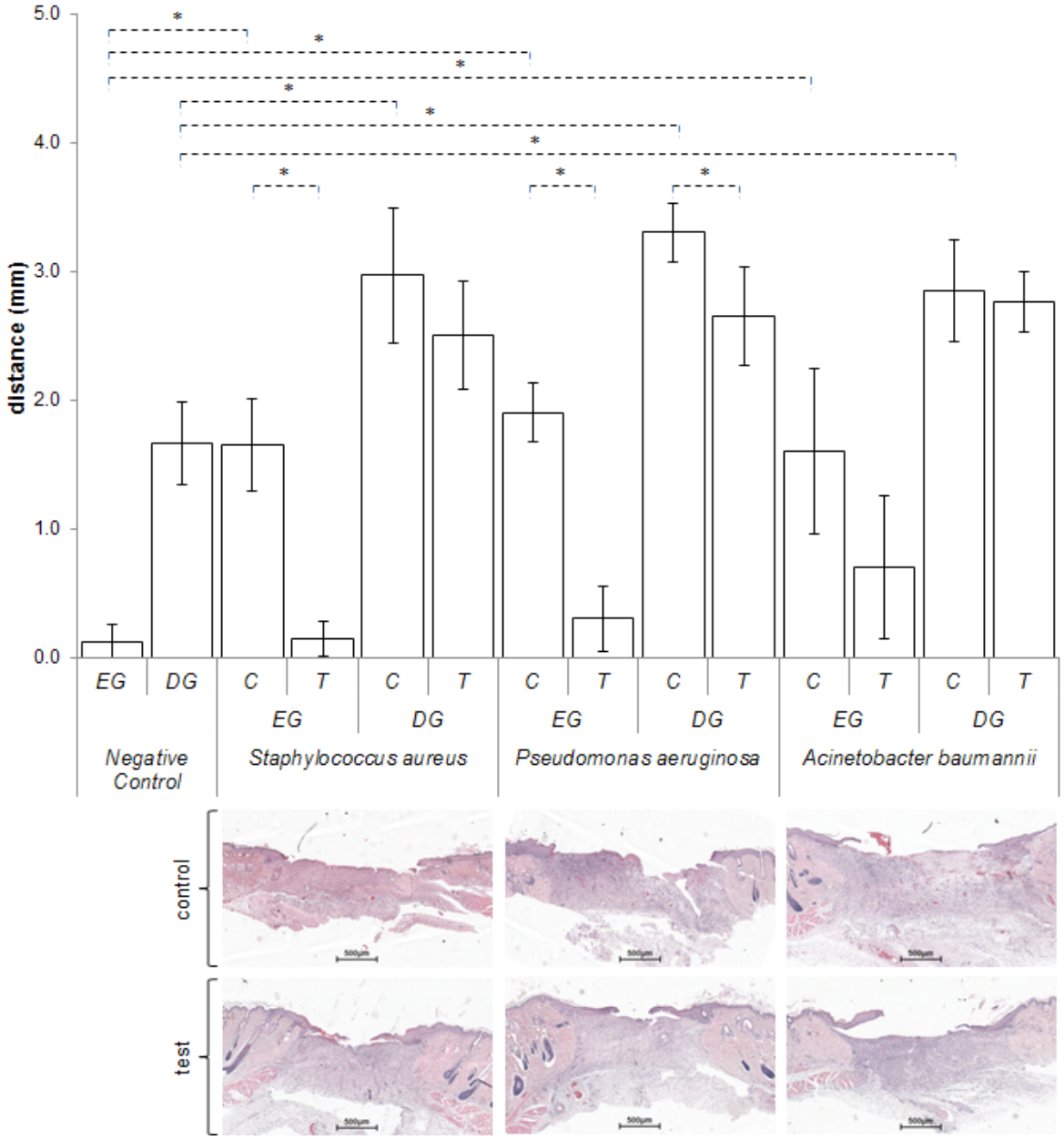


Figure B4.2-3 – Histological wound analysis in rats. Epithelial gap (EG) and dermal gap (DG) were measured in harvested ulcers. Significant differences were only observed in bacteriophage-treated wounds infected with *S. aureus* and *P. aeruginosa*. C, control, T, test; *P < 0.05.

The results of the histological study are presented in Figure B4.2-3. There was a statistically significant difference between the negative control group and all inoculated control subgroups for both EG (negative control, 0.11 ± 0.13 mm; *S. aureus*-inoculated control, 1.65 ± 0.36 mm, P < 0.01; *P. aeruginosa*-inoculated control, 1.90 ± 0.23 mm, P <0.01; *A. baumannii*-inoculated control, 1.60 ± 0.64 mm, P < 0.01) and DG (negative control, 1.67 ± 0.32 mm; *S. aureus*-inoculated control, 2.97 ± 0.52 mm, P < 0.01; *P. aeruginosa*-inoculated control, 3.30 ± 0.23 mm; *A. baumannii*-inoculated control, 2.85 ± 0.39 mm, P < 0.01). There was a statistically significant difference for EG between control and test subgroups in the *S. aureus*-inoculated (test, 0.14 ± 0.13 mm, P < 0.01) and *P. aeruginosa*-inoculated groups (test, 0.30 ± 0.25mm, P < 0.01). In DG, the difference between test and control subgroups only achieved statistical significance in the *P. aeruginosa*-inoculated group (test, 42.9 ± 10.2%, P = 0.02). There were no significant differences between control and test subgroups with regard to EP or DG in the *A. baumannii*-inoculated group.

Pig model

The results of the microbiological study are presented in Figure B4.2-4 A. Before treatment (*t0*), the average swab colony count for the *S. aureus*-inoculated, *P. aeruginosa*-inoculated, and *A. baumannii*-inoculated groups were 5.94 ± 0.69 log(cfu/swab), 4.81 ± 0.18 log(cfu/swab), and 3.61 ± 1.87 log(cfu/swab), respectively. There were no statistically significant differences between the test and control subgroups. After induction therapy (*t1*), there was a statistically significant difference in colony count in selective media between control and test subgroups in the *S. aureus*-inoculated [control, 6.02 ± 0.29 log(cfu/swab); test, 3.28 ± 1.84 log(cfu/swab); P = 0.02] and *P. aeruginosa*-inoculated groups [control, 4.81 ± 0.80 log(cfu/swab); test, 2.49 ± 1.46 log(cfu/swab); P = 0.03]. Although there was a tendency for microbial load reduction in average colony count for the *A. baumannii*-inoculated test and control subgroups [control, 2.52 ± 2.29 log(cfu/swab); test, 1.41 ± 1.65 log(cfu/swab)], the difference was not statistically significant. At day 4 after treatment initiation (*t4*), there was a significant difference in colony count between control and test subgroups in the *S. aureus*-inoculated [control, 5.93 ± 0.66 log(cfu/swab); test, 2.20 ± 1.14 log(cfu/swab); P < 0.01] and *P. aeruginosa*-inoculated [control, 4.81 ± 0.80 log(cfu/swab); test, 1.71 ± 1.05 log(cfu/swab); P = 0.02] groups. No such difference was observed for the *A. baumannii*-inoculated test and control subgroups.

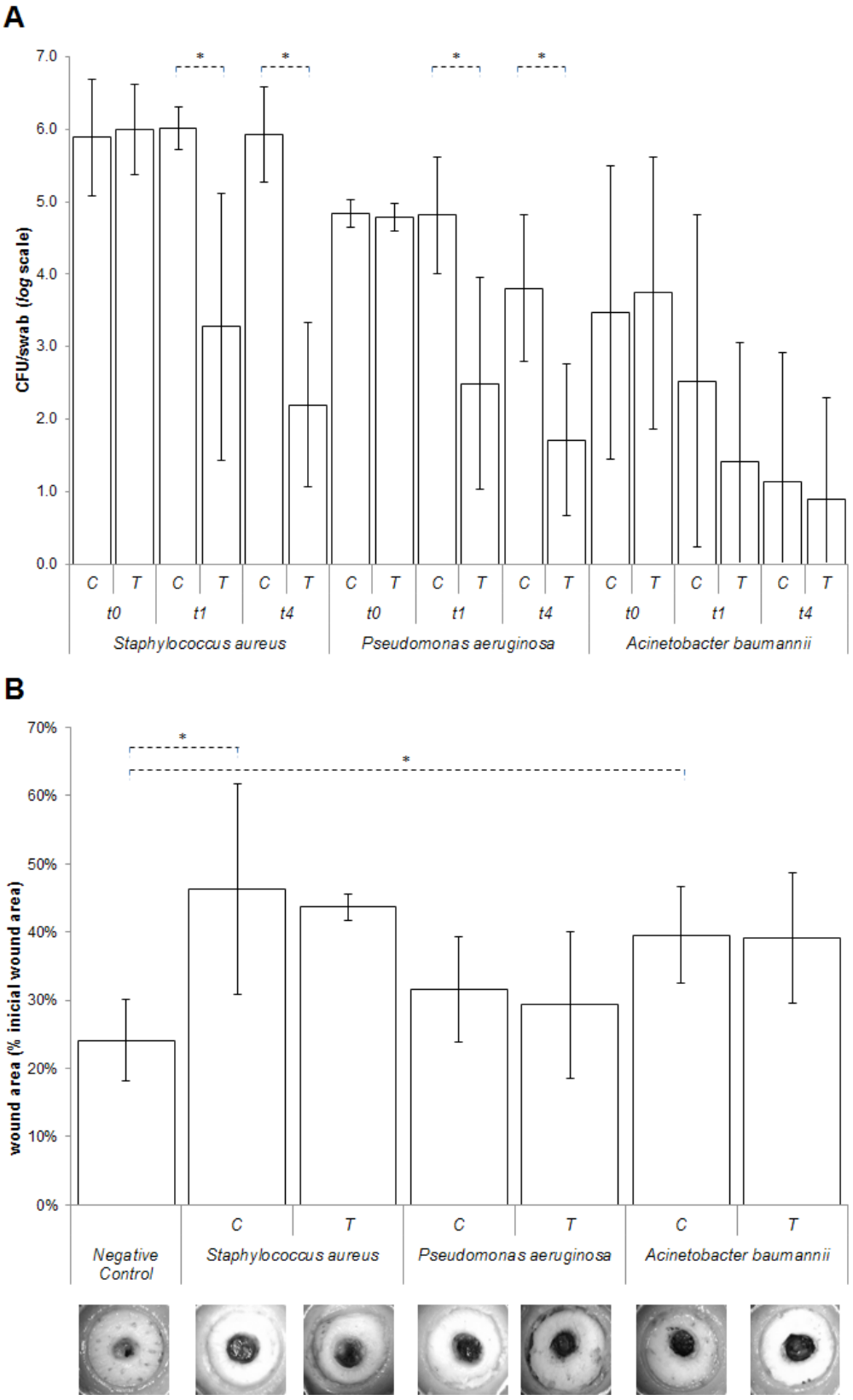


Figure B4.2-4 – (A) Average swab colony count in infected pigs. Wounds were swabbed at t0, t1, and t4, and the number of bacterial colony-forming units were compared between control (C) and test (T) conditions. The bacteriophage-treated wounds showed significantly lower counts for wounds infected with *S. aureus* and *P. aeruginosa*. *P < 0.05; (B) Wound closure kinetics in pigs. Bacteriophage treatment did not significantly decrease wound size for any of the three conditions. However, *S. aureus* and *P. aeruginosa* infections both resulted in larger significantly larger wounds. *P < 0.05.

The wound closure kinetics (planimetric) results are presented in Figure B4.2-4 B. Wound closure kinetics statistically differed between the negative control group (24.1 ± 6.0%) and the *S. aureus*-inoculated (46.3 ± 15.5%, P = 0.02) and *A. baumannii*-inoculated (39.6 ± 7.1%, P = 0.01) control subgroups, but this was not verified for the *P. aeruginosa*-inoculated control subgroup. Wound closure kinetics were not significantly different between any inoculated control and test subgroups for the three bacteria strains.

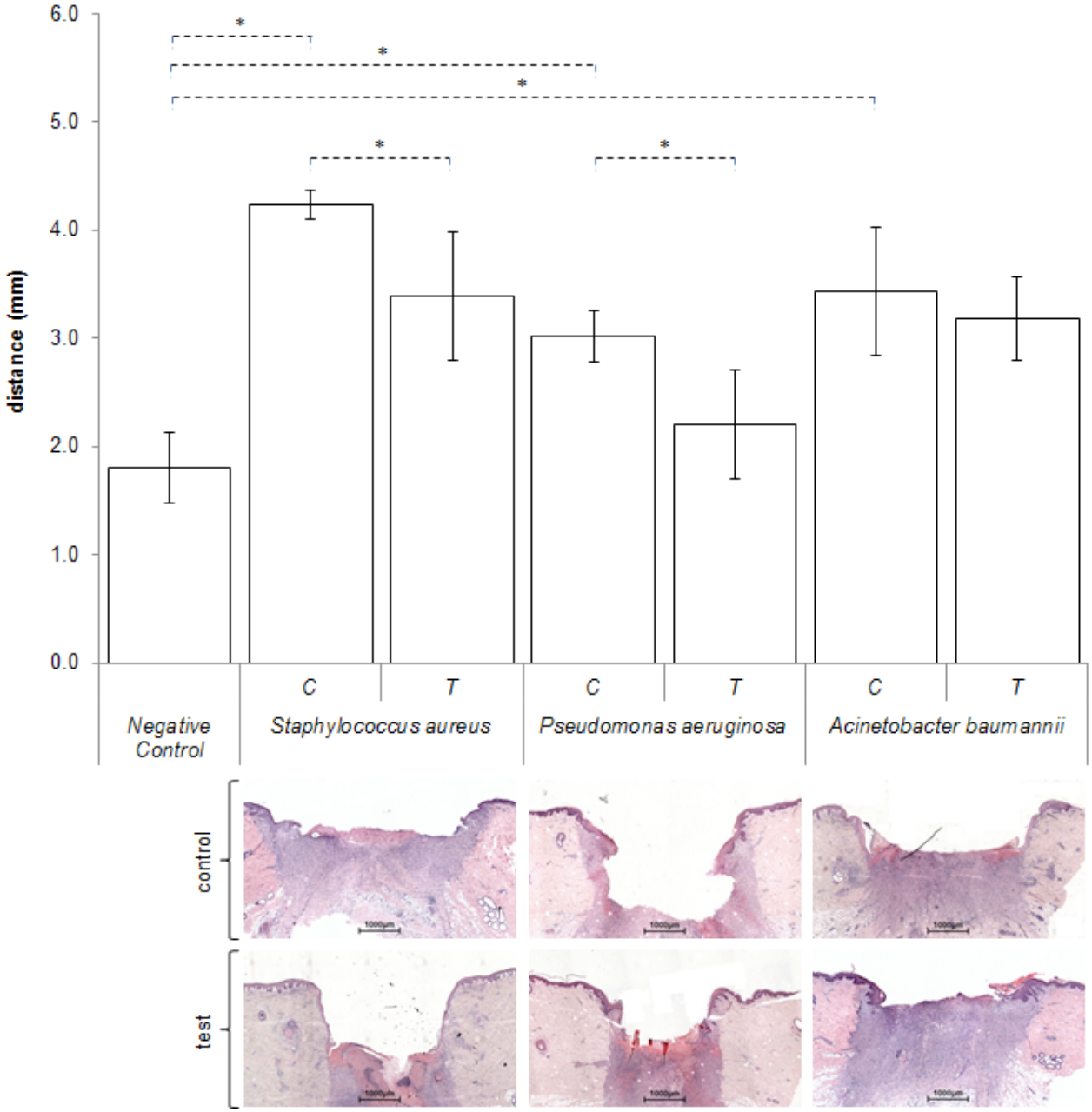


Figure B4.2-5 – Histological wound analysis in pigs. Epithelial gap (EG) values were measured in harvested wounds. Significant differences were only observed in *S. aureus* and *P. aeruginosa* bacteriophage-treated wounds. C, control, T, test; *P < 0.05.

The results of the histological study are presented in Figure B4.2-5. The analysis revealed a statistically significant difference with regard to EG between the negative control group and all the inoculated control subgroups (negative control, 1.80 ± 0.33 mm; *S. aureus*-inoculated control, 4.23 ± 0.14 mm, P < 0.01; *P. aeruginosa*-inoculated control, 3.02 ± 0.23 mm, P <0.01; *A. baumannii*-inoculated control, 3.43 ± 0.60 mm, P < 0.01). We also found statistically significant differences for EG between control and test subgroups in the *S. aureus*-inoculated (test, 3.38 ± 0.59 mm, P = 0.02) and *P. aeruginosa*-inoculated groups (test, 2.20 ± 0.05 mm, P = 0.02). No such difference was observed for *A. baumannii*-inoculated wounds.

Discussion

Current DFI treatment protocols employ debridement and systemic antibiotics (2), which are often ineffective in promoting wound healing (3). In addition, bacterial resistance to antibiotics renders these therapies less effective. Novel therapeutic regimens are needed to successfully treat DFIs. Although TATs are an attractive alternative, current formulations have not been successfully implemented for DFIs (7). The addition of lytic bacteriophages may enhance the utility of TAT agents; they are safe for human use (10) and can effectively combat drug-resistant bacteria (9). Although these bacteriophages have been used to treat acute bacterial infections (16), no study has assessed their ability to ameliorate chronically infected wounds.

Previously published studies have not assessed the effects of bacteriophage cocktails in chronic wounds; most examined outcomes after only a few hours of infection (16). In addition, most results came from burn models (33). We have investigated the ability of bacteriophages combined with debridement to improve microbiological, planimetric, and histological wound parameters in diabetic animal models.

Based on previous rodent studies (23) we knew that the bacterial colony counts in tissue cultured from infected wounds at t4 were, on average, 7.54 ± 0.19 log(CFU) per ulcer. We used high bacteriophage doses (10^8 to 10^9 pfu per administration), which yields a multiplicity of infection of 10 to 100. This initial dose is sufficiently in excess of the target bacterium population to cause reductions without the need for bacteriophages to replicate and complete their life cycle. This is in contrast with previous bacteriophage therapy studies (34) that employed relatively low bacteriophage doses and mainly relied on active therapy, which involves phage infection/replication cycles to reduce the target bacterium. These processes of active and passive bacteriophage therapy have been well described for in vitro and in vivo studies (35, 36).

All three outcomes were improved by bacteriophage treatment in animals that were infected with *S. aureus* and *P. aeruginosa*, but only bacterial reduction was observed in those infected with *A. baumannii*. This is potentially justified by a study (37) in which the presence of *Acinetobacter* spp. in a biofilm community was found to facilitate surface colonization by other species, namely Staphylococcus spp. Indeed, our microbiological data are in line with this finding. We determined that excess bacteria growing in non-selective media in *A. baumannii*-inoculated groups were primarily *Staphylococcus* spp.

Bacterial counts were assessed at t4, and colony counts were significantly different for *S. aureus* and *P. aeruginosa* test conditions compared to control. This difference was particularly pronounced for the latter. This finding is in agreement with our previously published optimization study (23). It is also in line with results reported by Fazli et al. (38), who used confocal laser scanning microscopy of clinical wound-biopsy specimens to demonstrate that the distance from *P. aeruginosa* aggregates to the wound surface was significantly greater than that of *S. aureus* aggregates, which led to an underestimation of the former in swab samples. This observation supports the possibility that factors intrinsic to each pathogenic bacterial strain can contribute to differences among studies that compare cultures grown from swabs and tissue samples.

Planimetric assessments revealed statistically significant differences between the control and test groups treated with *S. aureus* and *P. aeruginosa*. Although the same trend was observed for *A. baumanni*, the difference was not significant. These results were similar to the EG and DG measurements in harvested histological specimens. However, only the *P. aeruginosa* test group showed a significant difference. This was likely due the smaller standard deviation value in that group.

Although rodent models are useful, we wanted to assess the utility of bacteriophage treatment in pigs, which are considered the ideal large animal model for studying cutaneous disease (39).The results obtained in the rodent model were largely corroborated by experiments in swine. In both models, there was a significant reduction of bacterial counts at both time points (t1 and t4) for *S. aureus* and *P. aeruginosa* infections, but this was not verified for *A. baumannii* infections. A possible explanation for this difference is the low initial (t0) bacterial counts, which limited evaluation power. An alternative possibility for discrepant results between the two models is different host-microbe interactions between species, which may limit the establishment of bacterial infection in swine (40).

Although the planimetric results were not significantly different in the swine model, we did find differences among the negative control and *S. aureus* and *A. baumannii* control groups. Notably, the P. aeruginosa test group was not significantly different from the control group. It is possible that this was because that bacterial strain causes a deeper infection, and the damage was localized further beneath the dermis, resulting in a smaller ulcerated surface area (38). Despite the discrepant bacterial counts, there was no statistically significant difference between the *A*. *baumannii* and *S. aureus* group planimetries. However, both were significantly different from the negative control group. This may also be justified by the coaggregation of *Acinetobacter* spp. and other species, namely *Staphylococcus* spp.

We did observe significant results in the *S. aureus*-inoculated and *P. aeruginosa*-inoculated test animals with regard to EG measurements. However, the results for the *A. baumannii*-inoculated test group were not different from those of the control. A possible reason for this result is that the bacteria failed to successfully infect the wound.

There are several limitations inherent to the study design. We used swabs rather than biopsies to quantify bacterial counts. This method was chosen because we also evaluated planimetry and histology, both of which would have been influenced by biopsies. Moreover, the swab technique was previously optimized and shown to correlate with the invasive method for the rodent model (23). Secondly, we did not investigate resistance of bacterial strains to the bacteriophage cocktail, which may have played a role in the *A. baumannii*-inoculated groups. This variable will be studied in future investigations. Finally, we did not grow all the samples in non-selective media cultures; it was not feasible to simultaneously grow such a large number of samples in the lab without risking cross-contamination. Finally, the porcine experiments only included a single pig per group. Although this decreased the power of the statistical analysis, previously published findings suggest that the large degree of interindividual variability makes it necessary to make comparisons within individual animals (41).

To our knowledge, this is the first study to assess the microbiological, planimetric, and histological efficacies of topical bacteriophage therapy in animal models. It is important that the treatment was performed in conjunction with sharp debridement, as there is currently no clinical or experimental rationale for using TAT products in its absence (23). Debridement leads to a significant decrease in the resistance of the bioburden to TAT for up to 24 hours and enhances cicatrization (6). Collectively, the results of this study suggest that bacteriophage-containing TAT may be a viable treatment for DFIs, including infections caused by drug-resistant bacteria. Although additional studies are necessary, it may be an effective and novel therapeutic approach for addressing the serious problems associated with DFIs and other chronic skin and soft tissue infections.

References

1. Jeffcoate WJ, Harding KG. Diabetic foot ulcers. Lancet. 2003 May 3;361(9368):1545-51.
2. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. Clin Infect Dis. 2004 Oct 1;39(7):885-910.
3. Lipsky BA, Hoey C. Topical antimicrobial therapy for treating chronic wounds. Clin Infect Dis. 2009 Nov 15;49(10):1541-9.
4. Tascini C, Piaggesi A, Tagliaferri E, Iacopi E, Fondelli S, Tedeschi A, et al. Microbiology at first visit of moderate-to-severe diabetic foot infection with antimicrobial activity and a survey of quinolone monotherapy. Diabetes Res Clin Pract. 2011 Oct;94(1):133-9.
5. Mendes JJ, Marques-Costa A, Vilela C, Neves J, Candeias N, Cavaco-Silva P, et al. Clinical and bacteriological survey of diabetic foot infections in Lisbon. Diabetes Res Clin Pract. 2012 Jan;95(1):153-61.
6. Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, et al. Biofilm maturity studies indicate sharp debridement opens a time- dependent therapeutic window. J Wound Care. 2010 Aug;19(8):320-8.
7. Nelson EA, O'Meara S, Golder S, Dalton J, Craig D, Iglesias C. Systematic review of antimicrobial treatments for diabetic foot ulcers. Diabet Med. 2006 Apr;23(4):348-59.
8. Ansaldi M. Cell biology perspectives in phage biology. Front Biosci (Elite Ed). 2012;4:1823-9.
9. Rossney AS, Pomeroy HM, Keane CT. Staphylococcus aureus phage typing, antimicrobial susceptibility patterns and patient data correlated using a personal computer: advantages for monitoring the epidemiology of MRSA. J Hosp Infect. 1994 Mar;26(3):219-34.
10. Burrowes B, Harper DR, Anderson J, McConville M, Enright MC. Bacteriophage therapy: potential uses in the control of antibiotic-resistant pathogens. Expert Rev Anti Infect Ther. 2011 Sep;9(9):775-85.
11. Azeredo J, Sutherland IW. The use of phages for the removal of infectious biofilms. Curr Pharm Biotechnol. 2008 Aug;9(4):261-6.
12. Sulakvelidze A, Alavidze Z, Morris JG, Jr. Bacteriophage therapy. Antimicrob Agents Chemother. 2001 Mar;45(3):649-59.
13. McVay CS, Velasquez M, Fralick JA. Phage therapy of Pseudomonas aeruginosa infection in a mouse burn wound model. Antimicrob Agents Chemother. 2007 Jun;51(6):1934-8.
14. Soothill JS. Bacteriophage prevents destruction of skin grafts by Pseudomonas aeruginosa. Burns. 1994 Jun;20(3):209-11.
15. Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. Antimicrob Agents Chemother. 2005 Mar;49(3):1220-1.
16. Ryan EM, Gorman SP, Donnelly RF, Gilmore BF. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. J Pharm Pharmacol. 2011 Oct;63(10):1253-64.
17. Council of the European Communities. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Off J Eur Communities.L358:1-28.
18. Portuguese Agricultural Ministry. Portaria no. 1005/92 of 23 October on the protection of animals used for experimental and other scientific purposes. Diário da República I – Série B.245:4930-42.

19. Institute for Laboratory Animal Research. Guide for the care and use of laboratory animals. 8th ed. Washington DC, USA: National Academies Press; 2011.
20. Adams MH. Methods of study of bacterial viruses: Isolation of bacterial viruses. Bacteriophages. 1st ed. New York, USA: Interscience; 1959. p. 447-9.
21. Miller H. Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. Methods Enzymol. 1987;152:145-70.
22. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol. 2009;501:69-76.
23. Mendes JJ, Leandro CI, Bonaparte DP, Pinto AL. A rat model of diabetic wound infection for the evaluation of topical antimicrobial therapies. Comp Med. 2012 Feb;62(1):37-48.
24. Wu KK, Huan Y. Streptozotocin-induced diabetic models in mice and rats. Curr Protoc Pharmacol. 2008 Mar;Chapter 5:Unit 5 47.
25. Sullivan PK, Conner-Kerr TA, Hamilton H, Parrish-Smith E, Tefertiller C, Webb A. Assessment of wound bioburden development in a rat acute wound model - quantitative swab versus tissue biopsy. Wounds. 2004;16(4):115-23.
26. Weinstein MP. Diagnostic Technologies in Clinical Microbiology. In: Murray PR, Baron EJ, Jorgensen B, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology. 8th ed. Washington DC, USA: ASM Press; 2003. p. 183-1213.
27. Chapman GH. A single culture medium for selective isolation of plasma coagulating staphylococci and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation and the Stone reaction. J Bacteriol. 1946;51:409-10.
28. Brown VI, Lowbury EJ. Use of an improved cetrimide agar medium and other culture methods for Pseudomonas aeruginosa. J Clin Pathol. 1965 Nov;18(6):752-6.
29. Wareham DW, Gordon NC. Modifications to CHROMagar Acinetobacter for improved selective growth of multi-drug resistant Acinetobacter baumannii. J Clin Pathol. 2011 Feb;64(2):164-7.
30. Scherer SS, Pietramaggiori G, Mathews JC, Chan R, Fiorina P, Orgrill DP. Wound healing kinetics of the genetically diabetic mouse. Wounds. 2008;20(1):18-28.
31. Galiano RD, Michaels Jt, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen. 2004 Jul-Aug;12(4):485-92.
32. Hirsch T, Spielmann M, Zuhaili B, Koehler T, Fossum M, Steinau HU, et al. Enhanced susceptibility to infections in a diabetic wound healing model. BMC Surg. 2008;8:5.
33. Kumari S, Harjai K, Chhibber S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by Klebsiella pneumoniae B5055. J Med Microbiol. 2011 Feb;60(Pt 2):205-10.
34. Loc Carrillo C, Atterbury RJ, el-Shibiny A, Connerton PL, Dillon E, Scott A, et al. Bacteriophage therapy to reduce Campylobacter jejuni colonization of broiler chickens. Appl Environ Microbiol. 2005 Nov;71(11):6554-63.
35. Cairns BJ, Timms AR, Jansen VA, Connerton IF, Payne RJ. Quantitative models of in vitro bacteriophage-host dynamics and their application to phage therapy. PLoS Pathog. 2009 Jan;5(1):e1000253.
36. Hooton SP, Atterbury RJ, Connerton IF. Application of a bacteriophage cocktail to reduce Salmonella Typhimurium U288 contamination on pig skin. Int J Food Microbiol. 2011 Dec 2;151(2):157-63.

37. Simoes LC, Simoes M, Vieira MJ. Intergeneric coaggregation among drinking water bacteria: evidence of a role for Acinetobacter calcoaceticus as a bridging bacterium. Appl Environ Microbiol. 2008 Feb;74(4):1259-63.
38. Fazli M, Bjarnsholt T, Kirketerp-Moller K, Jorgensen B, Andersen AS, Krogfelt KA, et al. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol. 2009 Dec;47(12):4084-9.
39. Greenhalgh DG. Models of wound healing. J Burn Care Rehabil. 2005 Jul-Aug;26(4):293-305.
40. Mortensen BL, Skaar EP. Host-microbe interactions that shape the pathogenesis of Acinetobacter baumannii infection. Cell Microbiol. 2012 May 29.
41. Chvapil M, Gaines JA, Chvapil TA, Benson D, Tellez C. An optimal morphometric method for quantitating wound epithelization. J Surg Res. 1988 Mar;44(3):266-76.

Section C

General Discussion

Chronically infected wounds, such as DFIs, are a major source of morbidity. Treatment of these wounds involves considerable economic cost (1). In addition to debridement, effective treatment includes the use of high local concentrations of effective antimicrobials to suppress the growth of pathogenic bacteria (2). The efficacy of systemic antibiotic therapy is decreased by poor wound vascularization and the presence of biofilm-associated bacteria (3, 4). Systemic drugs may be unable to penetrate the affected areas at concentrations high enough to control the infection. Theoretically, this problem may be addressed by using a topical therapy capable of delivering high levels of antimicrobials to the infection site (5). In this particular clinical environment, lytic bacteriophages could be efficient antimicrobial agents because of their specificity and efficiency in lysing biofilm-associated pathogenic bacteria, including those that are MDR (6). Evidence from animal studies supports the hypothesis that topical BT is more effective than topically applied antimicrobials. Using a burn wound animal model, Kumari et al. (7) found that the level of protection from antimicrobials (i.e., silver nitrate and gentamicin) was lower compared with the protection provided by BT. BT has been, and continues to be, used for wound treatment in some countries (8).

A personalized (*sur-mesure* or bacteriophage bank-derived monophage) strategy is used in the countries where BT is applied to wounds (9). This strategy has theoretical benefits (10), and animal studies have provided evidence that it is effective (11), but we chose to develop a preformulated single cocktail (*prêt-à-porter*) strategy. One advantage of this strategy is that it allows early presumptive treatment, which maximizes the potential efficacy of the preparation. This concept was evaluated by Biswas et al. (12) using a mouse animal model of vancomycin-resistant *Enterococcus faecium* infection. They found that the mice who received bacteriophage treatment up to 5-hours post-infection recovered from the infection. However, only some of the mice who were treated after 5 hours recovered from the infection. There are also fewer problems associated with this model in terms of BT regulatory approval (9). It is a good fit with the combination of constraints imposed by drug regulation and typical medical practice (13).

In accordance with this model, we used a poly-bacteriophage cocktail for topical application. Our objective was to increase the utility of the formulation, and prevent the development of bacteriophage-resistant bacterial mutants during individual treatments (13). Our results have been positive, and similar to the results of recent animal (7, 14-19) and human (20-22) studies that used bacteriophage cocktails. This success is in contrast with the modest outcomes obtained when single bacteriophage topical preparations are used (23).

Bacteriophages are narrow-spectrum antibacterials and are often not effective against all strains, even within a single bacterial species (24). Therefore, the first challenge was to develop a formulation that contained the bacteriophage diversity needed to have a spectrum of activity that included the most relevant DFI bacterial pathogens. Even for a specific application, there are also geographic and epidemiological differences that may affect pathogen diversity, so information about the local epidemiology should be obtained. The results of Bourdin et al. (25) illustrate the importance of these geographical variations. They tested a cocktail consisting of 3 to 14 T4-like bacteriophages against four collections of diarrhea-associated *E. coli* isolates from different geographical origins, serotypes, pathotypes, epidemiological settings, and years of isolation. They found a maximum of 61% coverage against any of the four collections. We chose to overcome this issue by designing an epidemiological study to obtain information about bacterial targets and to aid in the assembly of a bacterial library against which to test the bacteriophages. We mostly isolated aerobic

gram-positive cocci from acute infections. Complex microbiotas that included gram-negative and anaerobic bacteria were found in the patients in our study. *S. aureus*, alone or as a component of a mixed infection, was the most frequently isolated pathogen. This result was similar to the results of studies performed in western countries (26, 27). But, there were some differences compared with the results from studies performed in India and other Asian countries (28). Our results reflect only the clinical and microbiological profiles of inpatients and outpatients in the Lisbon area. Care must be taken when these results are extended to other geographic areas, because patterns of microbial infections in DFUs vary worldwide.

Based on the results of the epidemiological studies, we selected *S. aureus*, *P. aeruginosa,* and *A. baumannii/calcoaceticus* as the bacterial targets. *S. aureus* (including MRSA) was selected because it was the most common cause of DFIs in our study population. We selected *P. aeruginosa* because it was associated with multi-drug resistance and antibiotic failure. *A. baumannii/calcoaceticus* was selected not only because it was associated with multi-drug resistance and antibiotic failure, but also because of its clinical relevance. The isolation of this pathogen is a marker of severe disease in hospitalized patients, but *A. baumannii* is not necessarily a more virulent organism (29). The medical community is concerned about its high rates of antibiotic resistance, especially in diabetic patients (30).

Technophage, S.A., previously isolated two *S. aureus* bacteriophages (F44/10 and F125/10), two *P. aeruginosa* bacteriophages (F770/05 and F510/08), and one *A. baumannii* bacteriophage (F1245/05) from environmental samples. These bacteriophages have been morphologically and genetically characterized. They have characteristics that increase their suitability for use for BT (31). They are incapable of lysogenically infecting bacteria and they do not encode bacterial virulence factor genes.

To assess their infectivity on target bacteria, these bacteriophages were tested against bacterial isolates that included 44 DFI isolates from the epidemiological study. The bacteriophage spot test procedure was used (32). This strategy did not differ from current BT cocktail preparation strategies. However, many environmental isolates that are sensitive in spot tests are unable to support plaque formation and have poor sensitivity (i.e., a very high bacteriophage concentration is needed for growth inhibition). The method and the concentrations that we used were acceptable (33). However, new strategies using highly quantitative methods based on the Bio-screen C microbial growth analyzer have been shown to be more stringent, compared with these traditional methods (34).

We formulated a bacteriophage cocktail with a sufficient spectrum of activity to cover the most relevant bacteria causing DFIs. However, as with any antimicrobial therapy, it was crucial to evaluate the in vitro and in vivo efficacy of the cocktail. This was necessary even after a topical application strategy was proved to be effective in multiple animal models of wound infection with common pathogens (35-37), including those targeted in our study. The clinical application of BT requires case-by-case experimental validation because outcomes vary by infectious agent, animal model of infection, and bacteriophage lytic potency. The current BT dosage regimens are mainly empirical or are based on complex mathematical models developed using parameter (adsorption constant, latent period, and burst size) values obtained in vitro (38). These models have been unable to predict the in vivo behaviour of bacteriophages and pathogens (39).

We developed a set of in vitro and in vivo studies and used the results to design a high-titer (10^9–10^{10} pfu/ml), multiple-dose, topical, BT protocol. In some respects, this pharmacologically-informed dosage regimen contrasted with the current protocols used in recent human clinical efficacy trials (20, 22), and in the clinical setting (40). These current protocols have produced modest results that are below expectations. One noteworthy example is the study published by Wright et al. (22). These investigators reported clinical and microbiological efficacy results for the first controlled double-blind

phase I/II clinical trial of a bacteriophage cocktail. The formulation contained six bacteriophages at 10^5 pfu and was applied topically to treat *P. aeruginosa*-associated chronic otitis after the site was prepared. Site preparation consisted of a thorough cleaning and debridement of the external auditory canal. Significant clinical improvements (measured by the subjective visual analogue scale) were reported for the treated group. However, those improvements were accompanied by only modest reductions in bacterial load (<1 log change, at specified time points up to 42 days). This BT model was based on an active therapy (41) characterized by a lower initial bacteriophage dosage. It is dependent on bacteriophage in situ replication to achieve a minimum inhibitory concentration capable of bacterial control. Our protocol was based on a passive therapy (41). Bacteriophages were supplied in numbers (high-titer dosing) sufficient to ensure that a minimum inhibitory concentration was exceeded. This peak in bacteriophage density was followed by a decline due to a combination of metabolism and decay. This decline can be avoided by repeated dosing. When this strategy is used, bacteriophages can still display in situ self-amplification (i.e., auto dosing) during antibacterial activity (41). Although not guaranteed to occur, this mechanism can provide a margin of safety toward attaining BT efficacy (42).

The desire for a high bacteriophage titer is based on the theoretical “multiplicity of 10 rule” (43, 44). If the goal is significant reduction in bacterial density, then approximately 10 bacteriophages should be available to adsorb to the average bacterium. We, along with others (41, 45), considered that a bacteriophage dose sufficiently in excess of the target bacterium population should be given to account for bacteriophage loss, dilution (associated with absorption and distribution), decay, and inefficiencies of bacteriophage adsorption to bacteria (e.g., inefficiencies in penetration into biofilms in vivo). This concept has been experimentally validated in studies of topical BT. Goode et al. (46) used lytic bacteriophages to reduce the contamination of chicken skin by *Salmonella* and *Campylobacter* spp. Kumari et al. (47) used a specific bacteriophage for the treatment of burn wounds infected with *K. pneumoniae* B5055. The investigators from both studies concluded that low-titer bacteriophage administration (IMs lower than 10) is unlikely to be successful.

We also used multiple doses divided into two phases, induction (every 4 hours for 24 hours) and maintenance (every 12 hours). The induction scheme was based on in vitro studies that found that an every 4 hours dose schedule is effective for limiting re-growth of all bacteria, except *A. baumannii.* The maintenance scheme was based on the current clinical standard used at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences. This multiple dose scheme was similar to the scheme used by Huff et al. (14). Using an animal model of *E. coli* respiratory infection, they found that multiple applications of bacteriophages are more effective than a single dose. The same observation was reported by Bardina et al. (15). Using different treatment schedules, they evaluated the effectiveness of a bacteriophage cocktail for the reduction of *Salmonella typhimurium* concentration in two animal models. Their results indicated that frequent treatment is required to achieve effective bacterial reduction over time. Other investigators have reported that compared with single dose treatment, multiple dose treatment does not improve the outcome. However, the principles and methodologies used in these studies were different from our studies. Vieira et al. (16) evaluated the ability of BT to inactivate *P. aeruginosa* in vitro and ex vivo (human skin). They found that the application of a second dose of bacteriophage does not increase the efficacy of the therapy. They used only one bacteriophage, and their results were similar to the results of our *A. baumannii* studies, in which we used only one bacteriophage. In in vitro experiments, Hall et al. (17) used *P. aeruginosa* and four bacteriophages to test whether simultaneous or sequential application of bacteriophage was more effective at

reducing bacterial densities and minimizing resistance emergence. Across different bacteriophage combinations, simultaneous application was consistently equal or superior to sequential application in terms of bacterial count reduction. There were also no differences in terms of minimization of resistance. These investigators did not use repeat applications of different bacteriophages, which is one important difference between their work and ours.

The design of our studies had some limitations. Wound healing outcome depends on the bacterial count, the species involved, and the presence of potentiating factors (e.g. biofilm). The outcome also depends on the intrinsic or acquired virulence of the organisms, the numbers of different species present, and the host immune response (48). We compared the efficacy of different bacteriophages against different planktonic bacterial species. Using metabolic activity as a measure of cell viability, we also examined efficacy against bacteria within established biofilms. However, we did not specifically study other outcome variables.

One major limitation of our studies was that we did not test surviving bacteria for bacteriophage resistance and retention of pathogenicity. The results of the in vitro studies indicated that regrowth was apparent at 5 hours, and more significantly, at 24 hours. This result was expected because of the high initial bacterial inoculum ($5x10^7$ cfu/ml). O'Flynn et al. (49) found resistance frequencies of 10^{-6} to 10^{-4} for single-bacteriophage and 10^{-6} for double-bacteriophage treatment in vitro. However, the evolution of bacteriophage resistance in vitro does not seem relevant to in vivo results. Bacteria replicate more slowly in in vivo conditions, and are challenged by environmental variability. This difference was illustrated by results obtained by Capparelli et al. (50). They reported an average resistance frequency of 10^{-8} for *S. aureus* treated with bacteriophages in vitro, but were unable to isolate any bacteriophage-resistant *S. aureus* strains in vivo. Moreover, even when BT does not clear the bacterial infection, it may indirectly impair growth by causing resistance mutations that are costly to fix, and that can reduce bacterial growth and virulence. Hall et al. (17) used a wax moth larvae infected by *P. aeruginosa* model. They found that resistance has a fitness cost. This result is similar to the results of other studies. Bacteriophage-resistant *E. coli* mutants that emerge during infections of mice and cattle are less virulent (32, 33).

Another study limitation was that the efficacy of the bacteriophage cocktail was not evaluated in a wound infected by multiple microorganisms. This limitation is important because in the epidemiological study, polymicrobial cultures were obtained from 83.7% of patients, and the isolation rate was 3.0 ± 1.4 bacteria per patient. In theory, various interference phenomena may occur (51), but the clinical outcomes of mono- and poly-microbial infections are similar (40). This outcome may occur because some therapeutic bacteriophages encode lytic enzymes that degrade the bacterial polymeric components (52). It may also occur as a result of bactericidal effects on specific pathogenic or co-aggregative species grown in a polymicrobial biofilm. We did not specifically study this issue, but in our animal studies we found that *A. baumannii* was a potential co-aggregator of the bacteria biofilm community. Contrary to expectations, F1245/05 (a therapeutic bacteriophage capable of reducing *A. baumannii* bacterial counts in vivo) was unable to limit the growth of *Staphylococcus* spp., and wound healing did not occur. The effect of BT on polymicrobial wound healing cannot be easily predicted. The outcome depends on complex interactions among the bacteriophages, the microbial species, and the infected host.

We did not address some aspects of the association between BT and the modulation of the host immune response, and between BT and cytotoxicity. These effects are important because they may condition the effectiveness of BT, and are often used as an argument against its use (10). It is well-accepted that intravenously administered bacteriophages may evoke a substantial immune response

(53), but few articles describe results for topical applications. Because the kinetics of bacteriophage action are much faster than the production of neutralizing antibodies, the development of neutralizing antibodies should not be a significant obstacle during the initial treatment of acute infections (54). We used prolonged BT schemes whose efficacy may be conditioned by this immune response. However, this effect may have been mitigated by the frequently repeated administration or by high local bacteriophage concentrations. The results from many studies indicate that in addition to pathogen eradication, BT exerts its effects by immune enhancement. The most expressive immune modulatory phenomenon is the enhancement of granulocyte phagocytosis (55). A reduction in bacterial count occurred in our in vivo studies. We did not specifically study the mechanisms, but a partial involvement of a weak immune response stimulated by the bacteriophage solution cannot be ruled out. The literature describing the results of cytotoxicity studies is also insufficient, particularly for wound models. The results from our rodent and porcine animal models clearly indicated that BT resulted in wound healing. However, we did not specifically evaluate cytotoxicity. This is important because minor interference by bacteriophage preparations could greatly affect clinical outcomes during the wound healing process.

There are currently no clinical or experimental rationale for the use of topical antimicrobials without debridement (56). Therefore, we did not evaluate the efficacy of BT without debridement. Using a lagomorph model, Seth et al. (57) found that topical BT was therapeutically ineffective in the absence of debridement. Also, BT should not be considered as a stand-alone therapy. It should be used in combination with approved treatments, especially with antibiotics. Chibber et al. (58) found that MR-10 lytic bacteriophage was effective for the resolution of hindpaw foot infection in diabetic mice. Co-therapy with linezolid was more effective for arresting the entire infection process in vivo.

In conclusion, antibiotic therapy with debridement is the standard first-line therapy for most DFIs. However, increasing numbers of patients present with antibiotic-resistant infections, and the identification and development of an alternative therapy is crucial. Our multi-faceted assessment of topical BT using methodologically different phases was a meaningful way to address this issue. These studies have enabled us to develop an antimicrobial product comprised of a combination of five lytic bacteriophages. We also developed a high IM (≥10) and multiple dose (every 4 hours for 24 hours) protocol for topical BT application. Our product and protocol represent a potentially effective therapeutic approach for treatment of diabetic wounds infected with different pathogens. The efficacy results obtained in our studies represent the first step in the development of a fully regulated human clinical trial that explores the potential for the use of BT for the treatment of DFIs and other chronic skin and soft tissue infections.

Future prospects

The results obtained in these experimental studies are not limited to BT. Based on the microbial isolates of the epidemiological study, we started a structured project that involves the collaboration of a network of national and international academic, clinical, and industry-related institutions with the objective of characterizing the microbiological isolates and their interactions in DFI. This project includes the genetic typing and phenotypic and genotypic evaluations of antimicrobial resistance patterns in the *S. aureus* and *P. aeruginosa* isolates, in addition to the assessment of their biofilm expressions and the characterization of their quorum sensing systems. Furthermore, although it was developed for BT, our wound infection model of diabetic rodents can be used to test other TAT agents.

This study used a practical approach to the design of a bacteriophage treatment protocol with potential efficacy in diabetic wound chronic infections. The approach did not focus on some aspects that could be targeted in future studies to reveal insights into the population dynamics of bacteriophage-bacteria interactions under conditions relevant to BT:

- The clinical application of topical BT in the context of DFIs would probably be more important in severe infections involving the deep layers of the foot, particularly the bone. Animal models of osteomyelitis are available (59), so future animal studies should be envisaged.

- In Western clinical practice, the topical application of bacteriophages in the absence of other systemic antibiotics would be highly unlikely. A synergistic effect has already been identified (60); however, systematic studies in this area have never been carried out, and bacteriophages are so specific in their actions that it is difficult to predict where these interactions may occur. Therefore, these interactions should be addressed in future in vitro and in vivo studies.

- In all studies, we found either the regrowth or the incomplete eradication of bacteria. Although the evolution of bacteriophage resistance in vitro does not seem relevant in vivo where bacteria replicate more slowly and are challenged by a greater set of environmental conditions (50), the biological basis (genotypic and/or non-heritable mechanisms) of these findings should be investigated in further studies.

- A practical clinical application of bacteriophage has to be developed. There is emerging evidence that NPWT devices enhance wound healing, although its effect on reducing the bacterial load is debatable (61, 62). Thus, the efficacy of bacteriophage preparations instilled into the wound using medical devices, particularly NPWT devices with an infusion port, should be investigated in appropriate, optimized animal models (63).

On the other hand, scientific discoveries must be translated into practical applications for the improvement of human health (64). Thus, the goal of this study is ultimately to transform BT into a realistic, everyday strategy for treating DFIs. We have maintained an open dialogue with the institutions responsible for regulating human trials in Portugal (Autoridade Nacional do Medicamento e Produtos de Saúde [INFARMED]) and abroad (EMA and US FDA), which have resulted in the need to conduct toxicology studies in a good laboratory practice (GLP) environment using batches of industrially produced bacteriophages. TechnoPhage, S.A. (which

holds the patents of the bacteriophages used in this study) and its commercial partners decided to outsource these services. If their results are suitable, we will be able to accomplish a fully regulated human trial. Furthermore, the knowledge gained about the biology and pharmacology of bacteriophages will be harnessed in the future development of new non-systemic formulations with potential applications in BT. For example, the application of inhalation technologies to BT has been one of the most recent advances within the field (65) and seems the next logical step.

References

1. Thomas S. Cost of managing chronic wounds in the U.K., with particular emphasis on maggot debridement therapy. J Wound Care. 2006 Nov;15(10):465-9.
2. Werdin F, Tennenhaus M, Schaller HE, Rennekampff HO. Evidence-based management strategies for treatment of chronic wounds. Eplasty. 2009;9:e19.
3. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. Clin Infect Dis. 2004 Oct 1;39(7):885-910.
4. Mendes JJ, Neves J. Diabetic foot infections: current diagnosis and treatment. The Journal of Diabetic Foot Complications. 2012;4(2):26-45.
5. Sibbald RG, Williamson D, Orsted HL, Campbell K, Keast D, Krasner D, et al. Preparing the wound bed--debridement, bacterial balance, and moisture balance. Ostomy Wound Manage. 2000 Nov;46(11):14-22, 4-8, 30-5; quiz 6-7.
6. Burrowes B, Harper DR, Anderson J, McConville M, Enright MC. Bacteriophage therapy: potential uses in the control of antibiotic-resistant pathogens. Expert Rev Anti Infect Ther. 2011 Sep;9(9):775-85.
7. Kumari S, Harjai K, Chhibber S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by Klebsiella pneumoniae B5055. J Med Microbiol. 2011 Feb;60(Pt 2):205-10.
8. Gorski A, Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Lobocka M, Fortuna W, et al. Bacteriophage therapy for the treatment of infections. Curr Opin Investig Drugs. 2009 Aug;10(8):766-74.
9. Pirnay JP, De Vos D, Verbeken G, Merabishvili M, Chanishvili N, Vaneechoutte M, et al. The phage therapy paradigm: pret-a-porter or sur-mesure? Pharm Res. 2011 Apr;28(4):934-7.
10. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. Bacteriophage. 2011 Mar;1(2):111-4.
11. Henry M, Lavigne R, Debarbieux L. Predicting in vivo efficacy of therapeutic bacteriophages used to treat pulmonary infections. Antimicrob Agents Chemother. 2013 Dec;57(12):5961-8.
12. Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, et al. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. Infect Immun. 2002 Jan;70(1):204-10.
13. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. Future Microbiol. 2013 Jun;8(6):769-83.
14. Huff WE, Huff GR, Rath NC, Balog JM, Donoghue AM. Bacteriophage treatment of a severe Escherichia coli respiratory infection in broiler chickens. Avian Dis. 2003 Oct-Dec;47(4):1399-405.
15. Bardina C, Spricigo DA, Cortes P, Llagostera M. Significance of the bacteriophage treatment schedule in reducing Salmonella colonization of poultry. Appl Environ Microbiol. 2012 Sep;78(18):6600-7.
16. Vieira A, Silva YJ, Cunha A, Gomes NC, Ackermann HW, Almeida A. Phage therapy to control multidrug-resistant Pseudomonas aeruginosa skin infections: in vitro and ex vivo experiments. Eur J Clin Microbiol Infect Dis. 2012 Nov;31(11):3241-9.
17. Hall AR, De Vos D, Friman VP, Pirnay JP, Buckling A. Effects of sequential and simultaneous applications of bacteriophages on populations of Pseudomonas aeruginosa in vitro and in wax moth larvae. Appl Environ Microbiol. 2012 Aug;78(16):5646-52.

18. Takemura-Uchiyama I, Uchiyama J, Kato S, Inoue T, Ujihara T, Ohara N, et al. Evaluating efficacy of bacteriophage therapy against Staphylococcus aureus infections using a silkworm larval infection model. FEMS Microbiol Lett. 2013 Oct;347(1):52-60.
19. Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophages phiMR299-2 and phiNH-4 can eliminate Pseudomonas aeruginosa in the murine lung and on cystic fibrosis lung airway cells. MBio. 2012;3(2):e00029-12.
20. Markoishvili K, Tsitlanadze G, Katsarava R, Morris JG, Jr., Sulakvelidze A. A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. Int J Dermatol. 2002 Jul;41(7):453-8.
21. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care. 2009 Jun;18(6):237-8, 40-3.
22. Wright A, Hawkins CH, Anggard EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clin Otolaryngol. 2009 Aug;34(4):349-57.
23. Skurnik M, Strauch E. Phage therapy: facts and fiction. Int J Med Microbiol. 2006 Feb;296(1):5-14.
24. Hyman P, Abedon ST. Bacteriophage host range and bacterial resistance. Adv Appl Microbiol. 2010;70:217-48.
25. Bourdin G, Navarro A, Sarker SA, Pittet AC, Qadri F, Sultana S, et al. Coverage of diarrhoea-associated Escherichia coli isolates from different origins with two types of phage cocktails. Microb Biotechnol. 2014 Mar;7(2):165-76.
26. Tascini C, Piaggesi A, Tagliaferri E, Iacopi E, Fondelli S, Tedeschi A, et al. Microbiology at first visit of moderate-to-severe diabetic foot infection with antimicrobial activity and a survey of quinolone monotherapy. Diabetes Res Clin Pract. 2011 Aug 6.
27. Citron DM, Goldstein EJ, Merriam CV, Lipsky BA, Abramson MA. Bacteriology of moderate-to-severe diabetic foot infections and in vitro activity of antimicrobial agents. J Clin Microbiol. 2007 Sep;45(9):2819-28.
28. Zubair M, Malik A, Ahmad J. Clinico-microbiological study and antimicrobial drug resistance profile of diabetic foot infections in North India. Foot (Edinb). 2011 Mar;21(1):6-14.
29. Mahgoub S, Ahmed J, Glatt AE. Underlying characteristics of patients harboring highly resistant Acinetobacter baumannii. Am J Infect Control. 2002 Nov;30(7):386-90.
30. Boyanova L, Mitov I. Antibiotic resistance rates in causative agents of infections in diabetic patients: rising concerns. Expert Rev Anti Infect Ther. 2013 Apr;11(4):411-20.
31. Skurnik M, Pajunen M, Kiljunen S. Biotechnological challenges of phage therapy. Biotechnol Lett. 2007 Jul;29(7):995-1003.
32. Armon R, Kott Y. A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. J Appl Bacteriol. 1993 Apr;74(4):490-6.
33. Kutter E. Phage host range and efficiency of plating. Methods Mol Biol. 2009;501:141-9.
34. Cooper CJ, Denyer SP, Maillard JY. Rapid and quantitative automated measurement of bacteriophage activity against cystic fibrosis isolates of Pseudomonas aeruginosa. J Appl Microbiol. 2011 Mar;110(3):631-40.
35. Soothill JS. Treatment of experimental infections of mice with bacteriophages. J Med Microbiol. 1992 Oct;37(4):258-61.

36. Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. Antimicrob Agents Chemother. 2005 Mar;49(3):1220-1.
37. Soothill JS. Bacteriophage prevents destruction of skin grafts by Pseudomonas aeruginosa. Burns. 1994 Jun;20(3):209-11.
38. Cairns BJ, Timms AR, Jansen VA, Connerton IF, Payne RJ. Quantitative models of in vitro bacteriophage-host dynamics and their application to phage therapy. PLoS Pathog. 2009 Jan;5(1):e1000253.
39. Weld RJ, Butts C, Heinemann JA. Models of phage growth and their applicability to phage therapy. J Theor Biol. 2004 Mar 7;227(1):1-11.
40. Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Fortuna W, Letkiewicz S, Szufnarowski K, et al. Clinical aspects of phage therapy. Adv Virus Res. 2012;83:73-121.
41. Abedon ST, Thomas-Abedon C. Phage therapy pharmacology. Curr Pharm Biotechnol. 2010 Jan;11(1):28-47.
42. Payne RJ, Phil D, Jansen VA. Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. Clin Pharmacol Ther. 2000 Sep;68(3):225-30.
43. Abedon ST. Kinetics of phage-mediated biocontrol of bacteria. Foodborne Pathog Dis. 2009 Sep;6(7):807-15.
44. Kasman LM, Kasman A, Westwater C, Dolan J, Schmidt MG, Norris JS. Overcoming the phage replication threshold: a mathematical model with implications for phage therapy. J Virol. 2002 Jun;76(11):5557-64.
45. Loc-Carrillo C, Wu S, Beck JP. Phage therapy of wounds and related purulent infections. In: Hyman P, editor. Bacteriophages in health and disease. 1st ed. Cambridge, USA: CAB International; 2012. p. 185-202.
46. Goode D, Allen VM, Barrow PA. Reduction of experimental Salmonella and Campylobacter contamination of chicken skin by application of lytic bacteriophages. Appl Environ Microbiol. 2003 Aug;69(8):5032-6.
47. Kumari S, Harjai K, Chhibber S. Topical treatment of Klebsiella pneumoniae B5055 induced burn wound infection in mice using natural products. J Infect Dev Ctries. 2010 Jun;4(6):367-77.
48. Kingsley A. The wound infection continuum and its application to clinical practice. Ostomy Wound Manage. 2003 Jul;49(7A Suppl):1-7.
49. O'Flynn G, Ross RP, Fitzgerald GF, Coffey A. Evaluation of a cocktail of three bacteriophages for biocontrol of Escherichia coli O157:H7. Appl Environ Microbiol. 2004 Jun;70(6):3417-24.
50. Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D. Experimental phage therapy against Staphylococcus aureus in mice. Antimicrob Agents Chemother. 2007 Aug;51(8):2765-73.
51. Chan BK, Abedon ST. Phage therapy pharmacology phage cocktails. Adv Appl Microbiol. 2012;78:1-23.
52. Hughes KA, Sutherland IW, Jones MV. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiology. 1998 Nov;144 (Pt 11):3039-47.
53. Merril CR, Scholl D, Adhya SL. The prospect for bacteriophage therapy in Western medicine. Nat Rev Drug Discov. 2003 Jun;2(6):489-97.
54. Sulakvelidze A, Alavidze Z, Morris JG, Jr. Bacteriophage therapy. Antimicrob Agents Chemother. 2001 Mar;45(3):649-59.
55. Gorski A, Miedzybrodzki R, Borysowski J, Dabrowska K, Wierzbicki P, Ohams M, et al. Phage as a modulator of immune responses: practical implications for phage therapy. Adv Virus Res. 2012;83:41-71.

56. Mendes JJ, Leandro CI, Bonaparte DP, Pinto AL. A rat model of diabetic wound infection for the evaluation of topical antimicrobial therapies. Comp Med. 2012 Feb;62(1):37-48.
57. Seth AK, Geringer MR, Nguyen KT, Agnew SP, Dumanian Z, Galiano RD, et al. Bacteriophage therapy for Staphylococcus aureus biofilm-infected wounds: a new approach to chronic wound care. Plast Reconstr Surg. 2013 Feb;131(2):225-34.
58. Chhibber S, Kaur T, Sandeep K. Co-therapy using lytic bacteriophage and linezolid: effective treatment in eliminating methicillin resistant Staphylococcus aureus (MRSA) from diabetic foot infections. PLoS One. 2013;8(2):e56022.
59. Patel M, Rojavin Y, Jamali AA, Wasielewski SJ, Salgado CJ. Animal models for the study of osteomyelitis. Semin Plast Surg. 2009 May;23(2):148-54.
60. Comeau AM, Tetart F, Trojet SN, Prere MF, Krisch HM. Phage-Antibiotic Synergy (PAS): beta-lactam and quinolone antibiotics stimulate virulent phage growth. PLoS One. 2007;2(8):e799.
61. Rutherford RB, Baker JD, Ernst C, Johnston KW, Porter JM, Ahn S, et al. Recommended standards for reports dealing with lower extremity ischemia: revised version. J Vasc Surg. 1997 Sep;26(3):517-38.
62. Weed T, Ratliff C, Drake DB. Quantifying bacterial bioburden during negative pressure wound therapy: does the wound VAC enhance bacterial clearance? Ann Plast Surg. 2004 Mar;52(3):276-9; discussion 9-80.
63. Morykwas MJ, Argenta LC, Shelton-Brown EI, McGuirt W. Vacuum-assisted closure: a new method for wound control and treatment: animal studies and basic foundation. Ann Plast Surg. 1997 Jun;38(6):553-62.
64. Lean ME, Mann JI, Hoek JA, Elliot RM, Schofield G. Translational research. BMJ. 2008;337:a863.
65. Ryan EM, Gorman SP, Donnelly RF, Gilmore BF. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. J Pharm Pharmacol. 2011 Oct;63(10):1253-64.

Annexes

Faculdade de Medicina de Lisboa

Avaliação do Pé Diabético

(Avaliação Clínica e Microbiológica)

Nº INTERNO: ___

Identificação

Nome: _____

NP: _____

- ☐ Associação Protectora dos Diabéticos de Portugal
- ☐ Hospital Curry Cabral
- ☐ Hospital Garcia de Orta
- ☐ Hospital de Santa Maria } CHLN
- ☐ Hospital de Santa Marta } CHLC
- ☐ Hospital de Santo António dos Capuchos
- ☐ AMBULATÓRIO
- ☐ INTERNAMENTO

Médico: _____

Data: ____/____/_____

Produto:

☐ zaragatoa ☐ biopsia ☐ curetagem ☐ aspirado

hora da colheita: :

Anamnese:

(preencher todos os que se apliquem)

idade: ____ anos **género:** ☐ masculino ☐ feminino **duração da diabetes:** ____ anos **HbA_{1c}:** ____%

co-morbilidades: ☐ hipertensão (TA >130/80mmHg) ☐ dislipidémia (LDL >100mg/dL, HDL<40mg/dL, TG >150mg/dL) ☐ tabagismo (≥1 cigarro/dia) ☐ cardiopatia isquémica (síndrome coronário agudo (EAM/AI), intervenção coronária (PTCA/CABG), exame compatível (ortigrafia/PE/outro)) ☐ insuficiência renal crónica (IRC estadio 4 (TFG <30mL/min/1,73m²), técnica dialítica crónica (HDDP), transplante renal)

☐ úlceras prévias (nº _____) ☐ amputação prévia

Tipo de Pé:

(escolher um de três)

☐ neuropático ⟷ ☐ misto ⟷ ☐ isquémico

ausência de sensibilidade protectora em 2 de 3 locais na superfície plantar do pé — ausência de pulso pedioso e tibial posterior

Classificação PEDIS:

(preencher apenas um grau por item, aquele que melhor se aplicar)

Perfusão

- ☐ **Grau 1** – ausência de sintomas ou sinais de Doença Vascular Periférica
  pulso pedioso e tibial posterior palpáveis *ou*
  índice tornozelo braço 0,9 a 1,1
- ☐ **Grau 2** – sintomas ou sinais de Doença Vascular Periférica
  claudicação intermitente *ou*
  índice tornozelo braço <0,9 (com pressão arterial no tornozelo >50mmHg) *ou*
  estudo *Doppler* dos membros inferiores compatível
- ☐ **Grau 3** – sintomas ou sinais de Isquémia Crítica
  pressão arterial no tornozelo <50mmHg

Extensão

área de úlcera (após desbridamento): _____ cm^2

***Depth* (profundidade)**

- ☐ **Grau 1** – úlcera superficial
  não penetrando qualquer estrutura além da derme
- ☐ **Grau 2** – úlcera profunda
  penetrando através da derme (envolvendo aponevroses, músculo ou tendão)
- ☐ **Grau 3** – envolvimento do osso ou articulação

Infecção

Grau 1 – ausência de sinais ou sintomas de infecção

- ☐ **Grau 2** – infecção envolvendo apenas a pele e tecido subcutâneo
  presença de ≥2 de 4:
  tumor (eritema ou tumefacção)
  rubor (eritema 0,5 – 2cm)
  dor (dor à palpação)
  calor (elevação da temperatura)
  exsudado purulento (espesso, opalescente a branco ou hemático)
- ☐ **Grau 3** – infecção com extensão na profundidade
  abcesso, osteomielite, artrite séptica ou fasceíte **ou**
  rubor / eritema >2cms
- ☐ **Grau 4** – infecção com sinais de sépsis
  presença de ≥2 de 4:
  frequência respiratória >20rpm (ou PaCO2 <32mmHg)
  leucócitos >12,000 cél/mL) ou leucopenia (<4,000 cél/mL)
  temperatura >38ºC ou <35ºC
  frequência cardíaca >90bpm

Sensação

- ☐ **Grau 1** – presença de sensibilidade protectora
- ☐ **Grau 2** – ausência de sensibilidade protectora
  ausência de sensibilidade protectora em 2 de 3 locais na superfície plantar do pé

Localização e Tempo de Evolução da Úlcera:

(desenhar a localização aproximada na figura)

tempo de evolução da úlcera: ± _____ semanas *ou*
± _____ meses

Antibioticoterapia:

(indicar todos os antibióticos utilizados na terapêutica, A – actual S – suspensa ≤3meses)

	A	S		A	S		A	S
amoxicilina + ácido clavulânico	☐	☐	ertapenem	☐	☐	clindamicina	☐	☐
flucloxacilina	☐	☐	meropenem	☐	☐	metronidazol	☐	☐
ceftriaxona	☐	☐	imipenem + cilastatina	☐	☐	vancomicina	☐	☐
ceftazidima	☐	☐	ciprofloxacina	☐	☐	linezolide	☐	☐
piperacilina + tazobactam	☐	☐	co-trimoxazol	☐	☐	_____	☐	☐

Termo de Consentimento Informado

O objectivo deste estudo é caracterizar clínica e microbiologicamente o pé diabético infectado. Faz parte deste estudo o preenchimento de um formulário com dados clínicos e a colheita de amostras para exame microbiológico, pelos Técnicos de Saúde Assistentes (Médicos, Enfermeiros ou Podologistas) sempre que haja indicação clínica (*i.e.* sempre que daí resulte benefício clínico para o doente). É adoptada a técnica menos invasiva (zaragatoa, biopsia, curetagem ou aspiração) adequada à situação clínica. Este estudo não traz qualquer risco adicional (*i.e.* não inerente ao procedimento de colheita já clinicamente necessário) ao doente. Está garantido o acesso a qualquer etapa do estudo, para esclarecimento de eventuais dúvidas.

O investigador principal é **JOÃO JOÃO DUARTE ALVES MENDES,** licenciado em Medicina com o número de ordem 44052, contactável no Serviço de Medicina Interna do Hospital de Santa Marta pelo telefone (+351) 213 594 237.

É garantida a confidencialidade e a liberdade da retirada de consentimento a qualquer momento. As informações obtidas serão analisadas em conjunto com a de outros doentes não sendo divulgado a identificação de nenhum participante deste estudo. Não há despesas pessoais para o participante em qualquer fase do estudo, incluindo exames e consultas. Também não há compensação financeira relacionada à sua participação. Se existir qualquer despesa adicional, ela será absorvida pelo orçamento da pesquisa.

Eu _____ acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo "**Caracterização Clínica e Microbiológica do Pé Diabético Infectado**".

Eu discuti com o _____ a minha decisão em participar neste estudo. Ficaram claros quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidade, prejuízo ou perda de qualquer benefício que possa ter adquirido ou no meu atendimento neste Serviço.

Assinatura do Médico

_____

Assinatura do Doente

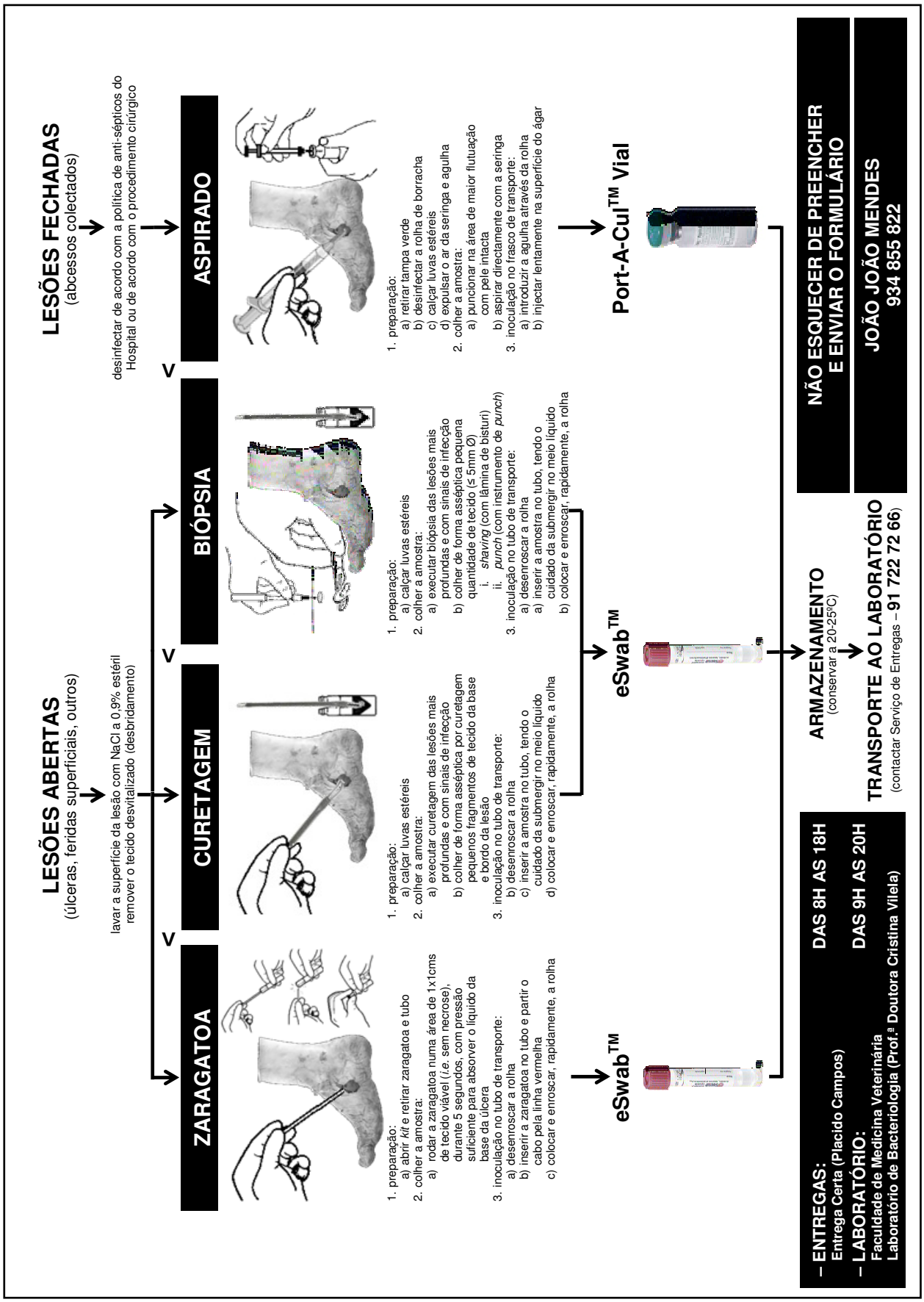
_____

Assinatura da Testemunha

_____

(para casos de doente analfabetos, semi-analfabetos ou portadores de deficiência auditiva ou visual)

B1.0-2


LESÕES FECHADAS
(abcessos colectados)
desinfectar de acordo com a política de anti-sépticos do Hospital ou de acordo com o procedimento cirúrgico
ASPIRADO
1. preparação:
a) retirar tampa verde
b) desinfectar a rolha de borracha
c) calçar luvas estéreis
d) expulsar o ar da seringa e agulha
2. colher a amostra:
a) puncionar na área de maior flutuação com pele intacta
b) aspirar directamente com a seringa
3. inoculação no frasco de transporte:
a) introduzir a agulha através da rolha
b) injectar lentamente na superfície do ágar
Port-A-Cul™ Vial
LESÕES ABERTAS
(úlceras, feridas superficiais, outros)
lavar a superfície da lesão com NaCl a 0,9% estéril
remover o tecido desvitalizado (desbridamento)
BIÓPSIA
1. preparação:
a) calçar luvas estéreis
2. colher a amostra:
a) executar biópsia das lesões mais profundas e com sinais de infecção
b) colher de forma asséptica pequena quantidade de tecido (≤ 5mm Ø)
i. shaving (com lâmina de bisturi)
ii. punch (com instrumento de punch)
3. inoculação no tubo de transporte:
a) desenroscar a rolha
a) inserir a amostra no tubo, tendo o cuidado da submergir no meio líquido
b) colocar e enroscar, rapidamente, a rolha
eSwab™
CURETAGEM
1. preparação:
a) calçar luvas estéreis
2. colher a amostra:
a) executar curetagem das lesões mais profundas e com sinais de infecção
b) colher de forma asséptica por curetagem pequenos fragmentos de tecido da base e bordo da lesão
3. inoculação no tubo de transporte:
b) desenroscar a rolha
c) inserir a amostra no tubo, tendo o cuidado da submergir no meio líquido
d) colocar e enroscar, rapidamente, a rolha
eSwab™
ZARAGATOA
1. preparação:
a) abrir kit e retirar zaragatoa e tubo
2. colher a amostra:
a) rodar a zaragatoa numa área de 1x1cms de tecido viável (i.e. sem necrose), durante 5 segundos, com pressão suficiente para absorver o líquido da base da úlcera
3. inoculação no tubo de transporte:
a) desenroscar a rolha
b) inserir a zaragatoa no tubo e partir o cabo pela linha vermelha
c) colocar e enroscar, rapidamente, a rolha
ARMAZENAMENTO
(conservar a 20-25ºC)
TRANSPORTE AO LABORATÓRIO
(contactar Serviço de Entregas – 91 722 72 66)
NÃO ESQUECER DE PREENCHER E ENVIAR O FORMULÁRIO
JOÃO JOÃO MENDES
934 855 822
– ENTREGAS:
Entrega Certa (Placido Campos)
DAS 8H AS 18H
– LABORATÓRIO:
Faculdade de Medicina Veterinária
Laboratório de Bacteriologia (Prof.ª Doutora Cristina Vilela)
DAS 9H AS 20H