UNIVERSIDADE DE LISBOA
Faculdade de Medicina Veterinária

ANTIMICROBIAL RESISTANCE OF THE UPPER RESPIRATORY TRACT COMMENSAL MICROBIOTA IN BOTTLENOSE DOLPHINS (*Tursiops truncatus*), UNDER HUMAN CARE

CATARINA AUGUSTA BÔTO MACHADO

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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,...

Adversity is like a strong wind. I don't mean just that it holds us back from places we might otherwise go. It also tears away from us all but the things that cannot be torn, so that afterward we see ourselves as we really are, and not merely as we might like to be.

Arthur Golden

Ao meu pai, Luís Filipe Alexandre Machado,

Agradeço tudo o que, de mim, é teu.

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Em primeiro lugar, queria agradecer à minha Mãe, Adosinda Machado. Uma mulher de força, coragem e determinação. Que receba o meu agradecimento - pelos valores que me transmitiu, beijinhos bons e sermões úteis. Por ter sofrido comigo (e por mim) e por me querer poupar dos males do mundo. Por me ter acompanhado, sem folgas. Pelo meu curso, pelas asas para voar e por, desde sempre, ter apoiado os meus sonhos, relembrando-me que mantivesse, sempre, os pés assentes na terra.
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Resumo

Resistência aos antibióticos da microbiota comensal do trato respiratório superior em golfinhos-roazes (*Tursiops truncatus*) mantidos sob cuidados humanos

Machado, C. B.

Universidade de Lisboa – Faculdade de Medicina Veterinária de Lisboa; 2014

As afeções respiratórias, especialmente a pneumonia bacteriana, são a principal causa de morte em golfinhos, tanto de vida livre como mantidos sob cuidados humanos. Os animais afetados por stresse, imunodeprimidos ou com outras condições subjacentes são mais suscetíveis a infeções por agentes oportunistas, frequentemente presentes no hospedeiro enquanto parte da microbiota comensal. Diversos microrganismos colonizadores do trato respiratório superior foram isolados a partir de um grupo de nove golfinhos saudáveis que vivem sob cuidados humanos no parque oceanográfico de entretenimento e educação Zoomarine - Mundo Aquático, S.A.. As estirpes identificadas pertencem às espécies bacterianas de *Escherichia coli, Enterococcus faecalis, Morganella morganii, Klebsiella oxytoca, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus aureus, Staphylococcus simulans* e *Staphylococcus delphini* grupo A. O presente estudo visou a avaliação das resistências destas estirpes relativamente a diferentes classes de antibióticos, tendo como base o método de difusão de disco e caracterização genotípica pela técnica de PCR. Nos isolados de *E. coli*, os genes de resistência a β-lactâmicos foram pesquisados através de PCR, para identificação de estirpes produtoras de β-lactamases (TEM, SHV, OXA-1, CTX-M, AmpC) assim como os genes de resistência a aminoglicosídeos (*aa*C*(3')-IV* e *aa*C*(6')-Ib*). Quanto aos isolados de estafilococos, foram pesquisados os genes de resistência *mec*A e *mec*C. De acordo com o método de difusão de disco, a maioria dos isolados demonstrou ser multirresistente, com uma percentagem de 76% de isolados resistentes a mais do que três classes de antibióticos, seguidos de 17% de estirpes resistentes e por uma pequena representação de isolados suscetíveis a todas as classes testadas, de 7%. Foram detetados genes de resistência em todos os isolados de *E. coli*, tendo sido mais comummente identificado o gene bla_{TEM}, seguido do bla_{OXA-1}, $bla_{CTX-M-15}$ e *aa*C*(6')-Ib,* e com menor frequência o bla_{DHA-1}. O gene *mec*A foi identificado numa estirpe de *S. aureus* e na única estirpe de *S. hominis.* O isolamento de estirpes multirresistentes na microbiota comensal do trato respiratório superior destes golfinhos-roazes é relevante na medida em que estes microrganismos são capazes de inativar um largo espectro de antibióticos, colocando limitações terapêuticas em caso de infeção. Associada à colonização do trato respiratório dos golfinhos, por estes organismos, surge a questão do potencial de transmissão e colonização entre estes animais e humanos.

Palavras-chave: golfinho-roaz, pneumonia, microbiota comensal, resistência antimicrobiana, genes de resistência, *S. aureus* meticilina-resistente

Abstract

Antimicrobial resistance of the upper respiratory tract commensal microbiota, in bottlenose dolphins (*Tursiops truncatus*), under human care

Machado, C.B.

Universidade de Lisboa – Faculdade de Medicina Veterinária de Lisboa; 2014

Respiratory affections, especially bacterial pneumonia, are a major cause of death in dolphins, both free-range individuals and those under human care. Animals affected by stress, immunocompromised or with underlying affections are more likely to be infected by opportunistic agents, usually present in the host in the commensal microbiota. Several colonizing microorganisms were recovered from the upper respiratory tract of nine healthy bottlenose dolphins, living under human care at the entertainment and educational oceanographic park Zoomarine - Mundo Aquático, S.A., Portugal. The isolated bacteria belonged to the species *Escherichia coli, Enterococcus faecalis, Morganella morganii, Klebsiella oxytoca, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus aureus, Staphylococcus simulans* e *Staphylococcus delphini* group A. Disk diffusion method and genotypic characterization through PCR were the techniques performed in order to evaluate the antimicrobial resistance of these strains, regarding different families of antibiotics. The presence of resistance genes to β-lactams was investigated in the *E. coli* isolates through PCR, in order to identify β-lactamases' producing strains (TEM, SHV, OXA-1, CTX-M, AmpC), as well as the resistance genes to aminoglycosides (*aa*C*(3')-IV* and *aa*C*(6')-Ib*). The *mec*A and *mec*C genes were investigated in the *Staphylococcus* spp. isolates. The results demonstrated that the majority of the isolates were multidrug-resistant, 76% of the isolates were considered clinically resistant to more than three antibiotic families (R>3), followed by 17% of resistant strains (1≤R≤3) and a small representation of 7% of fully susceptible bacteria (R=0). Resistance genes were detected in all the *E. coli* isolates, most frequently the bla_{TEM}, followed by bla_{OXA-1}, $bla_{CTX-M-15}$ and *aaC(6')-Ib* and less frequently the bla_{DHA-1}.. The *mec*A gene was identified in one *S. aureus* and in the *S. hominis* isolates. The isolation of multidrug-resistant bacteria from the commensal microbiota is relevant in that these microorganisms are capable of inactivating a wide spectrum of antibiotics, limiting the therapeutic options. Associated with the colonization of the respiratory tract of dolphins by these organisms, the question arises of the potential risk of colonization and transmission between these animals and humans.

Keywords: bottlenose dolphin, pneumonia, commensal microbiota, antimicrobial resistance, resistance genes, Methicillin-resistant *S. aureus*

Index

Index 7
Figures index 9
Tables and Charts index 10
Abbreviations List 11
Curricular Traineeship Report 13
I. Preface 15
II. Introduction 16
1. The Bottlenose dolphin (*Tursiops truncatus*) 16
1.1. The species 16
1.2. Husbandry and medical care 17
1.3. Respiratory anatomy and physiology 18
1.4. Commensal microbiota from the upper respiratory tract 19
1.5. Bacterial Pneumonia 21
2. Antibiotics 23
2.1. Routes of administration in dolphins 23
2.2. Mechanisms of antibacterial drug action 24
2.3. Antimicrobial resistance 29
2.3.2.4. Extended-Spectrum β-Lactamases (ESBLs) 34
3. Bacterial species 37
3.1. Enteric pathogens 37
3.2. Staphylococci 38
3.3. *Enterococcus faecalis* 38
III. Materials and Methods 39
1. Confirmation and characterization of the bacterial species 39
2. DNA Extraction 39
3. Polymerase Chain Reaction (PCR) 40
4. Antimicrobial Susceptibility Test 43
IV. Results and Discussion 44
1. Confirmation of the bacterial species 44

2. Antimicrobial Resistance Results 47
V. Conclusion 62
VI. Bibliographic References 63
VII. Attachments 72

Figures index

Figure 1 – Bottlenose Dolphin, adapted from Food and agriculture Organisation Species Identification Guide - Marine mammals of the World 16

Figure 2 – Dolphins' internal anatomy, adapted from www.imms.org 16

Figure 3 – Bottlenose dolphin interacting with dancer, during an exhibition at Zoomarine, Mundo Aquático, S.A., adapted from www.zoomarine.pt. 17

Figure 4 – Air sacs location, adapted from *Sisters of the Sinuses: Cetacean Air Sacs (*Reidenberg & Laitman, 2008). 18

Figure 5 – Pleuropneumonia in a bottlenose dolphin (post-mortem), adapted from *Two Cases of Bacterial Pneumonia in Bottlenose Dolphins (Tursiops gillii) at the Seoul Zoo, Korea*. *Pakistan Veterinary Journal*, Eo, K. & Kwon, O. (2011)..... 21

Figure 6 – Bottlenose dolphin being fed, adapted from: Getty, J. (2012) 23

Figure 7 – Mechanisms of action of the antibiotic families, adapted from: Johnson, K. (2011) 24

Figure 8 – Mechanisms of horizontal resistance gene transfer, adapted from Wang, J. (2006) 29

Figure 9 – Biological resistance mechanisms of bacteria, adapted from Wang, J. (2006) 30

Figure 10 - Methicilin-Resistant Staphylococcus spp. in Brilliance™ MRSA 2 agar (Oxoid®, Remel®, United Kingdom) 57

Tables and Charts index

Table 1 - β-lactamases classification according to Bush-Jacoby-Medeiros and Ambler scheme. Adapted from The continuous challenge of ESBLs, Perez et.al 2007 32

Table 2 - Hydrolysis profile of ESBLs and AmpCs, adapted from “Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals”; EFSA, 2011 33

Table 3 - Antimicrobial resistance results of the *Escherichia coli* isolates. 47

Table 4 - Resistance profile from the *Morganella morganii* strains 53

Table 5- Resistance Profile from the *Klebsiella oxytoca* strains 54

Table 6 - Resistance Profile from the *Enterococcus faecalis* strains 55

Table 7 - Compilation of the results from the staphylococci isolates 57

Table 8 - List of the Primers required for the Polymerase Chain Reaction protocols. Font: UL/FMV LRAB data base 73

Table 9 - Disk selection according to the bacterial species studied - disk diffusion method.. 75

Table 10 - Antimicrobial susceptibility test Results – inhibition zone diameter observed in the *E. coli* strains (In milimeters) 76

Table 11 - Antimicrobial susceptibility test Results – inhibition zone diameter observed in the *M. morganii* strains (In milimeters) 78

Table 12 - Antimicrobial susceptibility test Results – inhibition zone diameter observed in the *K.oxytoca* strains (In milimeters) 80

Table 13 - Antimicrobial susceptibility test Results – inhibition zone diameter observed in the *Enterococcus faecalis* strains (In milimeters) 81

Table 14 - Antimicrobial susceptibility test Results – inhibition zone diameter observed in the staphylococci strains (In milimeters) 82

Chart 1– Graphic representation of the bacterial species isolated per individual 44

Chart 2 - Graphic representation of the distribution of the recovered bacterial species 45

Chart 3 - Total number of resistances from the studied isolates 59

Abbreviations List

AK – Amikacyn

AMC – Amoxicillin + Clavulanic acid

AML- Amoxicillin

AMP – Ampicillin

AST - Antimicrobial susceptibility testing

bp - Base pairs

C – Chloramphenicol

CAZ – Ceftazidime

CB – Clinical breakpoints

CIP – Ciprofloxacin

CL – Cephalexin

CLSI - Clinical Laboratories Standards Institute

CN - Gentamycin

CNS – Coagulase negative staphylocci

CTX – Cefotaxime

CXM – Cefuroxime

DA - Clindamycin

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

dNTPs - Deoxyribonucleotide triphosphates

E - Erythromycin

ECOFF – Epidemiological cut-off values

EFSA – European Food Safety Authority

ENR - Enrofloxacin

ESBLs – Extended-Spectrum β-lactamases

ETP – Ertapenem

EUCAST – European Committee on antimicrobial susceptibility testing

FAO – Food and Agriculture Association of United Nations

FD – Fusidic acid

FEP – Cefepime

FFC – Florphenicol

FOS - Fosfomycin

FOX – Cefoxitin

G - Generation

HLAR - High level aminoglycoside resistance

I – Clinically intermediate (EUCAST)

IPM – Imipenem

IZD – Inhibition zone diameter

K – Kanamycin

KF – Cephalotin

LEV – Levofloxacin

LZD - Linezolid

MEM – Meropenem

MDR - Multidrug resistant

MgCl2 – Magnesium chloride

MRS - Methicillin-Resistant staphylococci

MRSA - Methicillin-Resistant Staphylococcus aureus

NA – Nalidixic acid

NaCl – Sodium Chloride

NSBLs – Narrow-spectrum β-lactamases

NWT – Non-wild type (EUCAST)

OX – Oxacillin

P- Penicillin

PABA - p-aminobenzoic acid

PBP - Penicillin-Binding Proteins

PIM – Pulmonary intravascular macrophages

PCR - Polymerase Chain Reaction

R – Clinically resistant (EUCAST)

RNA - Ribonucleic acid

S - Clinically susceptible (EUCAST)

Sa – *Staphylococcus aureus*

Sd – *S. delphini*

Shaem – *S. haemolyticus*

Shom – *S. hominis*

Ss – *S. simulans*

S3 - Sulfonamides compounds

STR - Streptogramins

SXT – Sulphonamide + trimethoprim

TE - Tetracycline

TOB - Tobramycin

URT - Upper respiratory tract

VA - Vancomycin

W - Trimethoprim

WT – Wild Type (EUCAST)

Curricular Traineeship Report

1. Zoomarine – Mundo Aquático, S.A.

I had the opportunity to work as an intern at Zoomarine – Mundo Aquático S.A, an entertainment and educational oceanographic park located in Guia, Albufeira, Portugal, for a period of six months, approximately 1056 hours. I was able to learn about several species, from marine mammals (bottlenose dolphins and pinnipeds), to reptiles (terrestrial turtles and semi aquatic turtles), tropical birds and birds of prey.

The veterinary team's role is essentially preventive - monitoring the animals in order to prevent the onset of disease is very important, since in marine mammals clinical signs are not often evident until the affection reaches an advanced stage. An early diagnosis is possible due to the close monitoring of the health status of these animals. For this reason, a monthly medical program is delineated and strictly followed. Several procedures such as blood screenings, blowhole and gastric cytologies, among others are carried out. As an intern, I had the opportunity to learn about the most prevalent medical issues of the species inhabiting the park. I was able to perform various laboratory techniques and gained experienced in interpreting the obtained results (haemograms, biochemistry profiles, faecal examination, blowhole exudate and gastric fluid examination, among others). As for intervention procedures, I had the opportunity to prepare and administrate drugs in different animal species, and I was trained how to proceed in emergency situations. I also assisted in avian and reptile surgeries, and had the opportunity to anaesthetise some animals, and performed several necropsies (birds, reptiles and fish). I developed skills in marine mammal ultrasound and radiology and took part in the preparation protocol for the birth of a bottlenose dolphin.

2. LRAB– Laboratory of Antimicrobial and Biocide resistance (FMV-UL)

I complemented my internship with four months at the LRAB in the Veterinary Medicine Faculty – University of Lisbon. I learned how to perform simple techniques, such as plating cultures on agar, as well as antimicrobial susceptibility testing techniques, such as minimal inhibitory concentrations by broth microdilution and disk diffusion methods. I also learned to perform the PCR method and how to extract DNA from Gram negative bacteria and staphylococci.

I. Preface

Respiratory affections, especially pneumonia, are a major cause of death in cetaceans, both free-range and under human care. Identifying the composition of commensal microbiota from the upper respiratory tract, in healthy dolphins, is important to evaluate the role of some bacterial species in the development of infections. Under normal conditions, commensal microbiota live within the host, without harming it. However, alterations of this balance can lead to microbial proliferation and subsequent infection. This is more likely to occur in stressed and immunocompromised individuals, as well as in animals with underlying affections, such as parasitism.

The current study arises as a continuation of the work developed by Fernandes (2012), who isolated and identified the composition of commensal microbiota of the upper respiratory tract, in bottlenose dolphins. With this aim, Fernandes collected respiratory exudate from nine healthy dolphins, living under human care at the entertainment and educational oceanographic park Zoomarine - Mundo Aquático, S.A. Several bacterial species were isolated and identified. The recovered strains were stored at -20ºC (Laboratory of Microbiology, Universidade de Trás-os-Montes e Alto Douro) for the last two years, before the beginning of the current study. Nowadays, a growing effort is being made to choose effective antimicrobial therapy when treating infections. The development of new antimicrobial resistances is currently of great concern, both in human and veterinary medicine. This is actually the result of selective pressure caused by the use of antimicrobial agents. In marine mammal medicine, the difficulty in choosing adequate antimicrobial therapy is associated not only with this, but also with the lack of information on the pharmacokinetics and pharmacodynamics of certain drugs. Therefore, the safety of some antimicrobial agents is uncertain and the therapy, in case of infection, relies on a limited range of antimicrobial agents. The present study aimed at the evaluation and characterization of the antimicrobial resistance of the commensal microbiota in the upper respiratory tract of bottlenose dolphins.

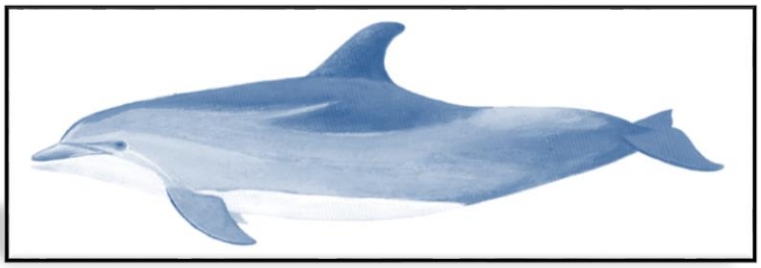
II. Introduction

1. The Bottlenose dolphin (*Tursiops truncatus*)

1.1. The species

The Order Cetacea is subdivided in two suborders - Odontoceti and Mysticeti, in which are comprised the toothed and baleen whales, respectively. The *Tursiops truncatus* species (Montagu, 1821, quoted by Jefferson, Leatherwood & Webber, 1993) belongs to the suborder Odontoceti and family Delphinidae, and is commonly known as bottlenose dolphin. (Jefferson *et al.*, 1993). This species is illustrated in Figure 1.

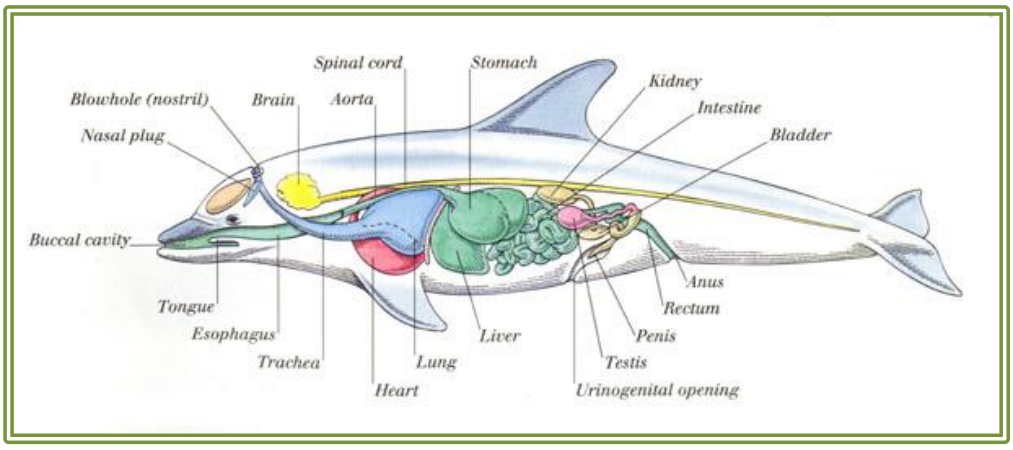
Figure 1 – Bottlenose Dolphin, adapted from Food and Agriculture Organisation Species Identification Guide - Marine mammals of the World (Jefferson *et al.*, 1993)



Bottlenose dolphins have a large falcate dorsal fin which is responsible for hydrodynamic stabilization. While the dorsal fin is supported by fibrous connective tissue, pectoral flippers are supported by the radius and ulna, carpals and phalanges. Dolphins have a smooth skin, covered by a thickened epidermis and, underneath the dermis, a layer of fat, called blubber, provides the necessary conditions for thermoregulation (Reidenberg, 2007; Uhen, 2007).

The internal organs of these animals (as shown in Figure 2) are similar to those of terrestrial mammals. In resemblance to ruminants, the stomach of dolphins is divided into three chambers (Reidenberg, 2007).

Figure 2 – Anatomy of the bottlenose dolphin, adapted from www.imms.org



1.2. Husbandry and medical care

The bottlenose dolphin is a charismatic species with great interest for public display (as seen in figure 3). Comprised in zoological collections, dolphins are preserved in zoos and oceanaria, therefore demanding special husbandry and medical care. (Wells, 2009)

Figure 3 – Bottlenose dolphin interacting with dancer, during an exhibition at Zoomarine, Mundo Aquático, S.A., adapted from www.zoomarine.pt.

Preventive medicine programs are thus designed to guarantee the welfare and maintain the health status of the individuals under human care. Husbandry and medical care of cetaceans advanced greatly in the past decades, due to the experience obtained from caring for these animals in zoological collections. Also, a growing effort to promote conservation of free-range dolphins, especially through the rescue of stranded animals and their rehabilitation, has been taking place. (Wells, 2009)

Wild animals are known to dissemble signs of disease, in order to survive longer in the wild, without attracting the attention of predators. The late appearance of clinical signs, which might not be evident until the disease reaches an advanced stage demands a tight monitoring of the health status of dolphins. (McBain, 2001)

In terms of medical care, obtaining a differential diagnosis requires gathering as much information as possible. A physical examination should be the basis of any diagnostic plan, in order to evaluate posture and behaviour, body condition and visual/palpable abnormalities, among other parameters (Varela, Schmidt, Goldstein, & Bossart, 2007). Afterwards, a blood sampling and screening allow the detection of quantitative and qualitative variations in the haemogram, biochemical profiles and hormonal quantification. Other biological samples such as faeces, urine, milk and gastric/respiratory cytologies might be analysed to achieve a final diagnosis. Bottlenose dolphins under human care are trained to collaborate voluntarily with veterinarians and trainers, in order to perform medical procedures (McBain, 2001; Wells,

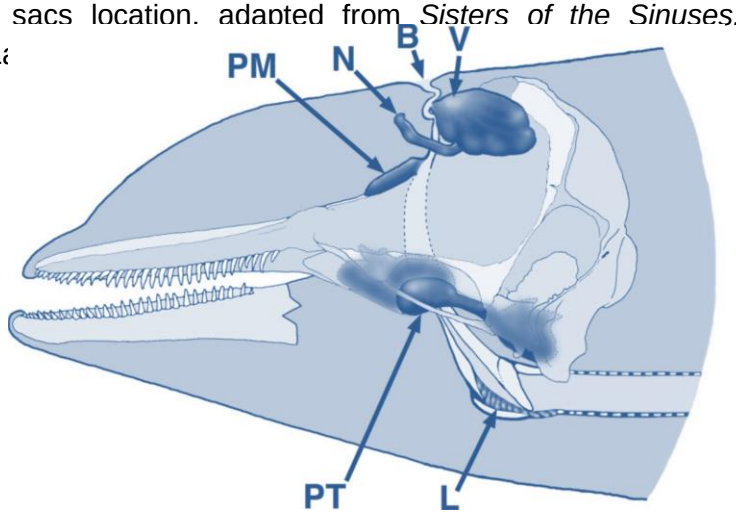
2009). Conditioned behaviour is not only beneficial for diagnostic purposes, but also for the animals' welfare, since this is a less invasive approach. (Wells, 2009)

1.3. Respiratory anatomy and physiology

Marine mammals were originally adapted for terrestrial living. According to Reidenberg (2007), the evolutionary process has led the return of these species to the oceans, meeting the challenges that an aquatic life demands. While pinnipeds spend some time ashore, for reasons such as breeding and resting, cetaceans are restricted to the marine environment (Jefferson *et al.*, 1993). This demands a series of adjustments of these species anatomical and physiological features.

Some general anatomical adaptive changes deserve further insight, especially those of the respiratory tract, which are more relevant to this document. A special feature of cetaceans is the presence of air sacs, illustrated in figure 4. These structures are necessary to retain large volumes of air, when diving, and withstand the high pressures and compression that deep diving requires (Cowan, 1968). Air sacs function as a reservoir of air, having an essential role in respiration, by controlling and conditioning air circulation, therefore facilitating gas exchanges. Vocalizing underwater is possible also due to the space created by the expansion of these structures, while the animals are immerged. Cetaceans have three types of air sacs, nasal, pterygoid and laryngeal sacs. Nasal sacs are homologous structures of the external nose of terrestrial mammals, and are characteristic structures of the Odontoceti suborder. There are three types of nasal sacs - vestibular, nasofrontal (anterior and posterior portion) and premaxillary. Their epithelium is keratinized, a feature related to vocal function of these particular sacs. The pterygoid sacs are referred to as "sinuses" and similarly to Laryngeal sacs, are present in the suborders Odontoceti and Mysticeti. (Reidenberg & Laitman, 2008)

Figure 4 – Air sacs location, adapted from *Sisters of the Sinuses: Cetacean Air Sacs* (Reidenberg & La



Labels: B – Blowhole; PT – Pterygoid sacs; L – Laryngeal sacs; Nasal sacs: PM – Premaxillary, N – Nasofrontal, V – Vestibular.

According to Bagnoli, Cozzi, Zaffora, Acocella, Fumero & Constantino (2011), the tracheo-bronchial tree has particular features to prevent its collapse, such as a rigid trachea, supported by complete and irregular cartilaginous rings. During deep diving, once the alveolar collapse occurs, the air flows from the alveoli to the upper respiratory ways, and becomes imprisoned due to special muscular sphincters which block its return to the lungs.

Another feature of these animals is their adapted thorax and lungs - the rib cage is jointed and collapsible and the lungs are unlobed and covered by an elastic and thick pleura (Reidenberg, 2007).

Alveolar macrophages, pulmonary interstitial macrophages and pulmonary intravascular macrophages (PIMs) are present in dolphins' lungs. PIMs exist within pulmonary small vessels of terrestrial mammals such as cattle, horses and cats. These cells remove potentially harmful particles in circulation, through active endocytosis. However, when releasing inflammatory mediators, these cells might influence the progression of an infection (Kawashima, Kuwamura, Takeya, & Yamate, 2004).

1.4. Commensal microbiota from the upper respiratory tract

According to Tlaskalová-Hogenová *et al.* (2004), the normal microbiota, also known as indigenous or resident microbiota, is the term that refers to communities of microorganisms (mainly bacteria and with less expression virus, fungi and protozoa) that colonize mucosal and skin surfaces of a healthy host. This harmonious co-existence is referred to as "commensalism" (from which the term *commensal microbiota* is original) and this concept translates the existence of a tight, beneficial relationship for both parts (Boman, 2000).

Due to the early host-bacteria interaction, body surfaces develop protective mechanisms, preventing the potential damage bacteria could inflict to the host (Tlaskalova-Hogenova *et al.*, 2004). Thus under normal circumstances the host tolerates the presence of these communities, and the immune system response is not triggered by them. However, persistent stressful circumstances may promote the development of functional alterations of the immune system, compromising this balance and overcoming protective host responses. It is estimated that over 1000 species of bacterial species could be identified in the commensal microbiota, and its composition depends on several factors such as age, race, hormones, diet, stress, sexual behaviour, medication, season, among others (Tlaskalova-Hogenova *et al.*, 2004; Avalos-Téllez, Suárez-Güemes, Carrillo-Casas & Hernández-Castro, 2010). Another important concept is that of transient microbiota. It refers to microorganisms that are present temporarily in the mucosal surfaces of the host (from days to several weeks). However, transient microbiota could be established within the host, becoming part of the resident microbiota. Microorganisms found in the commensal microbiota only during a limited period of time, are not considered resident microbiota. Pathogenic microorganisms are not usually associated to transient microbiota, and unless the composition of the resident

microbiota suffers a significant alteration there is not a risk of proliferating and causing an infection. (Lilly & Lowbury, 1978)

The microbiota of the respiratory tract is restricted to the URT structures. The trachea, bronchi and alveoli are sterile in healthy individuals. (Avalos-Téllez *et al.*, 2010)

The blowhole *sputum* is the biological sample used to identify the normal microbiota. To obtain accurate results, the individuals from the study group must be healthy and without antimicrobial medication for, at least, three weeks before the sampling. To ensure the health status of the individuals, prior to the study, a general examination and complementary exams, such as cytology, complete blood count and the evaluation of biochemistry parameters, ought to be performed. An early identification of alterations in the composition of the microbiota is essential to prevent the development of an infection. This enhances the importance of having preventive medicine programs in cetaceans under human care, comprising periodic samplings of the blowhole sputum (Varela *et. al*, 2007; Avalos-Téllez *et al*., 2010)

Morris *et al.* (2011), refers that free-range bottlenose dolphins are potential ecological reservoirs of pathogenic agents. Therefore, identifying the microorganisms that are part of the microbiota could be useful as an indicator of the ocean's health.

Several studies were performed in cetaceans, in order to describe the normal microbiota of the URT. Lima, Rogers, Acevedo-Whitehouse and Brown (2011) concluded that microbial communities were nearly identical in free-range and under human care dolphins. In 2006, Buck, Wells, Rhinehart and Hansen recovered the following microorganisms from bottlenose dolphins inhabiting the Coastal Gulf of Mexico and Atlantic Ocean waters: vibrios (36%), enteric bacteria (17.5%), unidentified pseudomonads (11%), yeasts (6.3%), staphylococci (5.8%), and streptococci/enterococci (2.2%).

Morris *et al.* (2011) isolated and identified 20 different bacterial species and 10 fungal species in free-range dolphins inhabiting two estuaries located in the south-eastern Atlantic Coast of the United States of America. Here, *Plesiomonas shigelloides, Aeromonas hydrophila, Escherichia coli* and *Pseudomonas fluorescens* were the most frequently isolated Gram negative bacteria, while *Clostridium perfringens, Bacillus* spp. and coagulase negative *Staphylococcus* were the most frequently isolated Gram positive bacteria. Higgins (2000) has listed, in his review, the following microorganisms recovered from the URT of cetaceans: *Brucella* spp., *Erysipelotrix rhusiopathiae*, *Staphylococcus delphini*, *Salmonella* spp., *Streptococcus* spp., *Proteus mirabilis*, *Vibrios alginolyticus* and *Clostridium perfringens*.

Despite some bacterial species recovered from healthy dolphins are considered part of the normal microbiota, in case of respiratory infection different bacterial species were identified. Some microbial agents, such as enteric bacteria, *Pseudomonas* spp. and *Staphylococcus* spp., were recovered from dolphins with pneumonia, suggesting that commensal agents might also assume a pathogenic behaviour. (Avalos-Téllez *et al.*, 2010).

1.5. Bacterial pneumonia

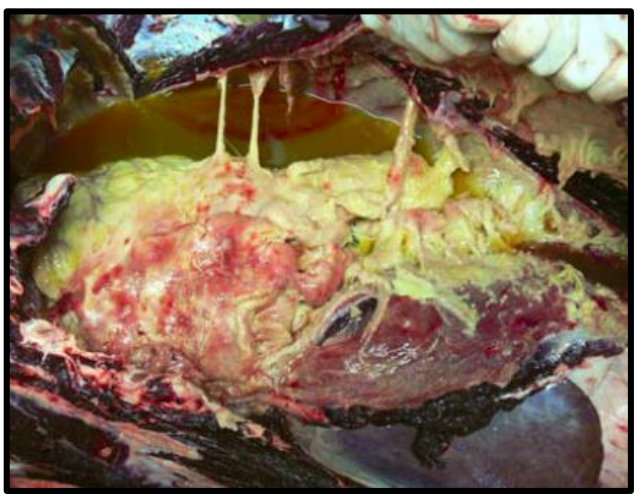
According to Prescott, Harley & Klein (2002) infectious processes arise when the ability of the host to preserve normal function is compromised in the presence of specific microorganisms or their products. There are two types of pathogens, primary agents and opportunistic agents. Primary agents cause disease most frequently by direct interaction, from host to host or less commonly indirectly, through contact with infected surroundings or environment. Infected soil, water or food may also be in the origin of a disease, and vectors and fomites may play a role in spreading those agents.

Opportunistic agents live in a tight relationship with the host, under normal conditions. However, under specific circumstances such as immunosuppression, are capable of assuming a pathogenic behaviour. Parasitism is one of the underlying causes associated with the development of bacterial diseases in dolphins (Avalos-Téllez, 2010).

Pathogenic bacteria are often isolated from the respiratory tract of cetaceans, being pneumonia a major cause of mortality in these animals, not only under human care but also free-range dolphins (Jeraj & Sweeney, 1996). A necropsy finding can be seen in figure 5. The anatomy of the upper respiratory tract of cetaceans is considered to play a role in the development of pneumonia, due to the some particularities. The inexistence of structures, such as filters or turbinates, and the rapid exchanges of large air volumes that occur when they reach the water surface, promote the entrance of pathogenic microorganisms and their colonization of the URT (Reidenberg & Laitman, 2008; Bagnoli *et al.*, 2011).

It has been commonly observed that infectious processes are usually localized, in these species (Eo & Kwon, 2011). Potentially dangerous inhaled particles are not expelled, which is the opposite of what happens in terrestrial mammals. Instead, these particles are trapped and retained, triggering oedema and cellular infiltration of the bronchial mucosa. This reaction promotes the formation of abscesses and necrosis. The consequences are either dense fibrous nodule or scar formation. (Cowan, 1968)

Figure 5 – Pleuropneumonia in a bottlenose dolphin (post-mortem), adapted from: Eo, K. & Kwon, O. (2011)



Dolphins under human care, inhabiting large pools, are exposed to water containing faecal matter in suspension and chemicals, such as chlorine (Wells, 2009). The risk of aspirating water increases during air exchanges and could lead to the development of an infection, as a result of the entrance of these compounds in the blowhole. Likewise, free-range dolphins are exposed to environmental contaminants and to a wide range of pathogenic microorganisms (Cowan, 1968).

Once an infectious process is diagnosed, it is of great importance to proceed with the identification of the bacterial species present in the URT. The microbial communities suffer modifications during an infection, and studying their composition might help to predict the progression of a disease (Lima *et al.*, 2011).

Bacterial pneumonia in cetaceans is usually associated to pathogenic bacterial species, such as *Aeromonas hydrophila, Aerobacter spp., Erysipelothrix rhusiopathiae, Staphylococcus aureus, Streptococcus* Group D*, P. mirabilis, E. coli* and *Pseudomonas aeruginosa*. However, these microorganisms are commonly found as part of the URT normal microbiota (Cusik & Bullock, 1973; Venn-Watson, Jensen & Ridgway, 2011).

In the *post-mortem* examination, pulmonary abscesses are often observed in animals that died of pneumonia. The most frequently isolated agents, in these findings, are *S. aureus* and *P. aeruginosa*, and with less expression, some Gram-negative bacteria (Lima *et al.,* 2011). Avalos-Téllez *et al.* (2010) also mentions the role of *Edwarsiella* spp., *Klebsiella* spp*., Salmonella* spp*., Vibrio spp., Aeromonas* spp*., Pseudomonas* spp*.* and *Pasteurella* spp*.* in the development of this potentially fatal disease. Higgins (2000) refers the occurrence of bronchopneumonia associated to *Streptococcus zooepidemicus* and *β-haemolytic Streptococcus.*

Clinical signs of pneumonia include depression, decreasing appetite, unusual posture, elevated respiratory rate, putrid odour from the exhaled air and coughing (Higgins, 2000; Eo & Kwon, 2011). These signs are not often noticeable until the disease reaches an advanced stage. The respiratory rate elevation, in case of pneumonia, arises when a large portion of pulmonary tissue is irreversibly deteriorated. (McBain, 2001)

A combination of clinical, laboratory and complementary diagnostic tools should be used to delineate an adequate therapeutic strategy. Besides the observation of the posture and behaviour of the animal, a blood examination (through evaluation of haematology and serum biochemistry parameters) and a blowhole exudate culture should be carried out in order to confirm a respiratory infection. Other complementary exams might provide further information, such as bronchoscopy, ultrasonography, thoracentesis, and radiography techniques (Avalos-Téllez *et al.*, 2010).

While awaiting for the routine culture and susceptibility results, a first line of therapy should be initiated. It is preferable to use systemic antibiotics, with a broad spectrum and a good activity against Gram-negative bacteria (Dunn, Buck & Robeck, 2001). According to Walsh &

Gearhart (2001), third-generation cephalosporins are ideal antimicrobial agents in a first approach, especially when in association with an aminoglycoside, since these families of antibiotics have a synergistic effect. However, Patterson & Bonomo (2005) refer the appearance of improved resistance genes encoding Extended-Spectrum enzymes, capable of inactivating 3rd G cephalosporins. Therefore, these antibiotics should be used with caution, after performing a susceptibility testing and confirming their efficiency against the pathogenic agents.

2. Antibiotics

The therapeutic approach of respiratory infectious diseases, as previously referred, comprises the administration of antibiotics. There is a lack of information concerning the use of several drugs and drug combinations in marine mammals, therefore caution is needed when using any medication for the first time (Stoskopf, Willens & McBain, 2001)

2.1. Routes of administration in dolphins

According to the severity of an infection, different options are available, concerning the routes of administration in dolphins. Yet, practical considerations and anatomical adaptations of this species impose limitations and challenge the administration of therapeutic agents (Stoskopf *et al*., 2001).

In severe cases, injectable agents are preferred. Adequate concentrations in the body tissues are achieved more efficient and rapidly, through parenteral routes. Intramuscular administration is often an option for calves that are not yet trained to cooperate with the trainers through voluntary behaviour, for calves with an exclusively milk diet and depressed animals refusing food. This administration route should be used with caution in dolphins to properly inject the solution into the muscular tissue and not in the subcutaneous blubber, at the risk of not achieving inhibitory concentrations and an adequate distribution to body tissues (Walsh & Gearhart, 2001). Dolphins that are eating well are good candidates for oral administration of antibiotics (as seen in figure 6), however this route demands a higher frequency of administration.

Figure 6 – Bottlenose dolphin being fed, adapted from: Getty, J. (2012)

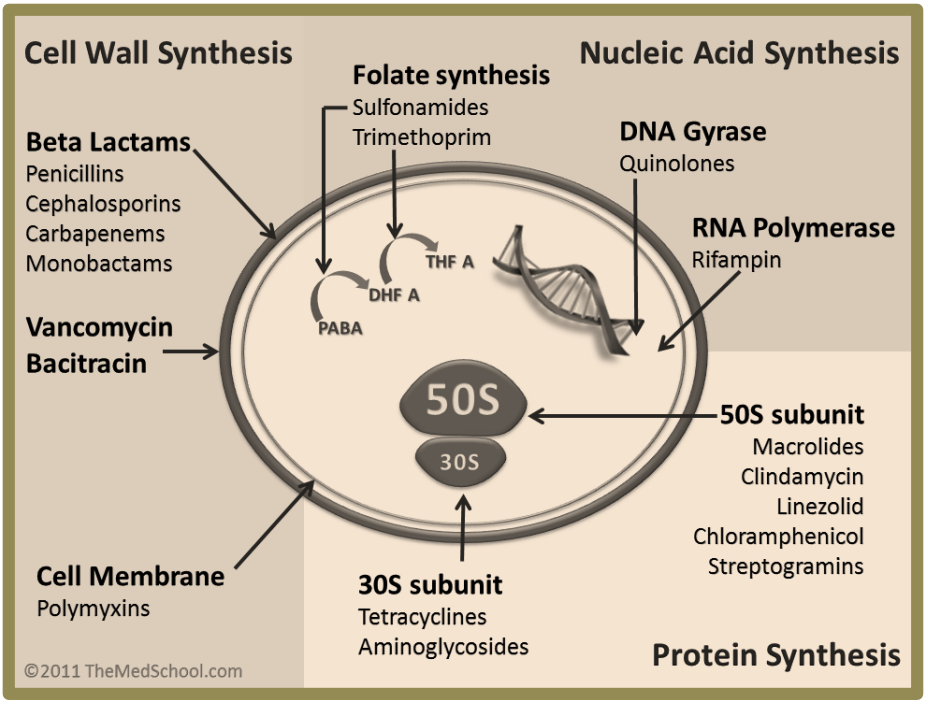
The effect of this route of administration can be compromised in case of diarrhoea, vomiting or other gastrointestinal absorption problems. Intra-tracheal route is not commonly used, however it is another option available, in dolphins. (Stoskopf *et al.*, 2001)

2.2. Mechanisms of antibacterial drug action

According to Prescott *et al.* (2002) chemotherapeutic agents are divided into two categories: bacteriostatic and bactericidal. The bacteriostatic antimicrobial drugs inhibit bacterial growth reversibly, which means that when the administration of the agent ceases, the microorganisms might return to growth. Therefore, its action is influenced by the immune response of the host. The bactericidal agents eliminate the microorganisms but in lower concentrations may only exert static action on the pathogens. Their activity might also differ depending on the target bacterial species.

These pharmaceutical agents can be divided based on their mechanisms of action, as shown in figure 7.

Figure 7 – Mechanisms of action of the antibiotic families, adapted from: Johnson, K. (2011)



2.2.1. Cell wall synthesis inhibition

Antimicrobial agents that interfere with the bacterial cell wall synthesis present a high selectivity to bacteria, not damaging the host cell.

2.2.1.1. Penicillins

Penicillins were the first β-lactam antibiotics to be discovered, and are derivatives of the 6-aminopenicillanic acid. An important feature of this type of antimicrobial agents is the

presence of a β-lactam ring on their structure (Prescott *et al.*, 2002; Alanis, 2005). The mechanism of action of penicillins relies on binding to several different proteins in the cell wall of bacteria. Its bactericidal action is primarily due to the inhibition of penicillin-binding proteins (PBPs) thus interfering with the peptidoglycan biosynthesis.

Penicillins are divided into penicillins (penicillin G and V), aminopenicillins (ampicillin, amoxicillin), penicillinase-resistant penicillins (methicillin, oxacillin) and antipseudomonal penicillins (carbenicilin, ticarcillin and piperacillin).

Aminopenicillins and antipseudomonal penicillins can be administered in association with a β-lactamase inhibitor, such as clavulanic acid or sulbactam. (Prescott *et al.*, 2002)

2.2.1.2. Cephalosporins

Similarly to penicillins, cephalosporins are also β-lactam antimicrobial agents, and likewise possess the β-lactam ring in their structure. These bactericidal antibiotics are separated accordingly to their spectrum, so far into four generations. While first-generation cephalosporins have a significant effect against Gram-positive bacteria, the second generation drugs are equally efficient against Gram-negative and Gram-positive microorganisms. As for third-generation cephalosporins, these agents are most effective inhibiting Gram-negative bacteria. (Alanis, 2005)

Examples: 1st Generation cephalosporins: cefazolin, cephalexin; 2nd Generation: cefoxitin, cefuroxime; 3rd Generation: cefotaxime, ceftazidime; 4th Generation: cefepime

2.2.1.3. Vancomycin and teicoplanin

These bactericidal antibiotics are structurally similar and share the same mechanism of action. However, these agents are exclusively approved for human use.. Vancomycin and teicoplanin interfere with the peptidoglycan regular synthesis, weakening the bacterial cell wall structure. Rising resistances of *Enterococcus* spp*.* strains to vancomycin have become prevalent, and are currently of great importance in human nosocomial infections. The target microorganisms of these antibiotics are Gram positive bacteria, such as staphylococci, enterococci and streptococci, among others (Levine, 2006).

2.2.1.4. Carbapenems

These antimicrobial agents also belong to the β-lactam family of antibiotics, and are efficient in the presence of β-lactamases including AmpC and extended spectrum β-lactamases. Imipenem, meropenem and doripenem are powerful broad spectrum agents, only available for human use, similarly to vancomycin and teicoplanin. Carbapenems are effective against Gram-positive, Gram-negative and anaerobic bacteria. Ertapenem has, however, a narrower spectrum of activity, since it is not effective against *P. aeruginosa* and *Enterococcus* spp*.* infections (Hellinger & Brewe, 1999; Zhanel *et al.*, 2007).

2.2.1.5. Monobactams

Aztreonam was the first monobactam marketed, and is often used as an alternative to aminoglycosides or third generation cephalosporins as a part of an empiric therapy (Brogden & Heel, 1986). This agent binds to the penicillin-binding protein 3 of susceptible Gram-negative pathogens, however is ineffective against Gram-positive and anaerobic bacteria. Because of its narrow spectrum, it is used commonly in association with other antimicrobial agents. (Finberg & Guharoy, 2012)

2.2.1.6. Polymixins

Polymyxin B sulfate and colistin are polymixins used in Gram-negative bacterial infections when microorganisms such as *P. aeruginos*a and those from the Enterobactereacea family are involved. These antimicrobial agents are often a therapeutic option in cases of multi-resistant infections caused by Gram negative bacteria (Evans, Feola & Rapp, 1999).

2.2.2. Protein synthesis inhibitors

Another mechanism of action that some antibiotics possess of interfering with the bacterial growth is the binding with the ribosome 30S or 50S subunit of the target bacteria, and thus inhibiting the protein synthesis. This compromises essential mechanisms for the bacteria, such as aminoacyl-tRNA binding, peptide bond formation, mRNA reading, and translocation (Prescott et al., 2002).

2.2.1.7. Antibiotics that inhibit 30s subunit

2.2.1.7.1. Aminoglycoside antibiotics

Aminoglycosides are bactericidal agents active against Gram negative and Gram positive bacteria. These antibiotics differ greatly in structure from each other, despite having a cyclohexane ring and amino sugars in their structure, in common. They interfere directly with the protein synthesis of the target microorganisms, similarly to tetracyclines. Some examples of aminoglycosides are gentamycin, tobramycin, amikacin and kanamycin (Alanis, 2005).

2.2.1.7.2. Tetracyclines

This broad-spectrum bacteriostatic antibiotic family is known for having a four-ring structure with side chains attached. These agents inhibit the protein synthesis of the target microorganisms. The antimicrobial agents bind to the 30S ribosomal subunit and prevent the binding of aminoacyl-RNA molecules to the ribosomal A site (Alanis, 2005).

2.2.1.8. Antibiotics that inhibit 50s subunit

2.2.2.1. Erythromycin and other macrolides

The macrolide family is known to contain a 12/22-carbon lactone ring attached to one or more sugars. Erythromycin is a bacteriostatic agent, mostly effective against Gram-positive and it is the most frequently used antibiotic from the macrolide family. It interferes in the protein synthesis through the inhibition of the peptide chain elongation, as a result from the agent's binding to the 23S rRNA on the 50S ribosomal subunit (Alanis, 2005).

2.2.2.2. Chloramphenicol

Chloramphenicol is a broad spectrum bacteriostatic antibiotic, with a similar mechanism of action to erythromycin. This is however a very toxic antimicrobial agent therefore it must be used with caution (Alanis, 2005).

2.2.2.3. Linezolid

Oxazolidinones are antimicrobial agents efficient against Gram-positive pathogens, including multiresistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant streptococci and vancomycin-resistant enterococci (Shinabarger *et al.*, 1997). These agents are approved for human use only. Their mechanism of action is based on targeting an early phase of protein synthesis, interfering with the binding of N-formylmethionyl-tRNA to the bacterial ribosome (Shinabarger, 1999).

Marchese and Schito (2001) mention the linezolid's ability to affect virulence factors and its post antibiotic effect, especially when combined with other antimicrobial agents.

2.2.2.4. Streptogramins

Streptogramins are divided into two types of compounds, streptogramins A (pristinamycin I and virginamycin S) and streptogramins B (pristinamycin II and virginamycin M). These agents exhibit a synergistic inhibition of bacterial growth. Bacteriostatic when used separately, these agents become bactericidal when combined (Vannuffel & Cocito, 1996).

2.2.3. DNA synthesis inhibitors

The inhibition of the nucleic acid synthesis is a less common mechanism of action that some antibiotics have by compromising the bacterial DNA replication. These agents have an irreversible action, whereas those altering the metabolism of other polymers cause a reversible inhibition of bacterial multiplication. (Prescott et al., 2002)

2.2.3.1. Quinolones and Fluoroquinolones

These broad-spectrum bactericidal antimicrobial agents contain a 4-quinolone ring and inhibit the bacterial DNA gyrase or topoisomerase II and topoisomerase IV, compromising the regular DNA processes of replication, repair, transcription and bacterial chromosome separation during division, among other phases. The target microorganisms are mostly enteric pathogens and some Gram-positive bacteria (Alanis, 2005). Examples of these antimicrobial agents are nalidixic acid, cinoxacin and oxolinic acid. Fluoroquinolones are an improved version of these antimicrobial agents and result from adding fluoride to these previous compounds and include agents such as enrofloxacin, ciprofloxaxin and levofloxacin (King, Malone & Lilley, 2000).

2.2.4. RNA synthesis inhibitors

2.2.4.1. Rifampin

This antimicrobial agent has a broad-spectrum activity, based on a specific mechanism of action – the bacterial RNA polymerase inhibition. It inhibits Gram-positive bacteria and mycobacteria, and is efficient even at low concentrations (Wehrli, 1983).

2.2.5. Folic acid synthesis inhibitors

Some antibiotics are able to interfere with metabolic pathways (antimetabolite drugs).

2.2.5.1. Sulfonamides or Sulfa Drugs

These were the first antimetabolites developed associated to therapeutic success, and are inhibitory competitors of *p*-aminobenzoic acid (PABA) for incorporation into folic acid. The target microorganisms are not then able to survive with decreased concentrations of this compound, which is essential for the cell synthesis. Eukaryotic cells obtain their folates from the animals' diet, therefore these antimicrobial agents interfere specifically with the prokaryotic cells which makes them selectively toxic, thus safe for the host. Currently, antimicrobial resistance to sulfonamides is a growing concern (Hitchings, 2012).

2.2.5.2. Trimethoprim

Trimethoprim's mechanism of action is based on the interference with the dihydrofolate to tetrahydrofolate (active folic acid) reduction. In this way, the agent inhibits the target microorganisms. It is efficient against most Gram-positive aerobic cocci and some Gram-negative aerobic rods (Gleckman, Blagg & Joubert, 1981).

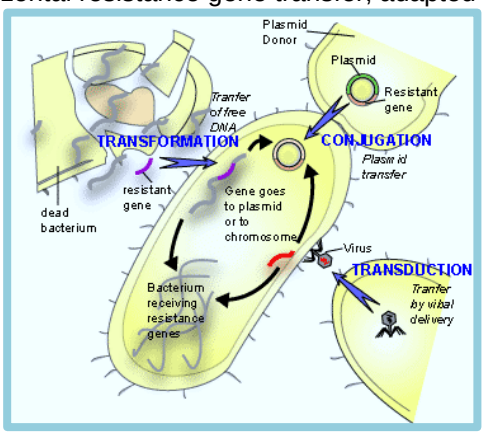
2.3. Antimicrobial resistance

Antibiotic resistance is perceived as a major emergent problem, in both veterinary and human medicine. Bacteria are adaptable microorganisms and therefore have the ability to adjust to hostile conditions, developing resistance mechanisms even to new antimicrobial agents (Alanis, 2005). Since antibiotics' introduction in 1940, a massive employment of these agents influenced microbial genetic ecology, promoting extensive bacterial natural selection and intense evolutionary pressure (Mazel & Davies, 1999). Despite being a complex relationship, it is accurate to say that antimicrobial resistance is a result of the excessive antibiotic use for the last sixty years, which resulted in the development of resistant and multiresistant bacterial strains, compromising the efficiency of antimicrobial agents and subsequently the infection's therapeutic success (Cars & Nordberg, 2004; Alanis, 2005).

The consequences of this worldwide problem are high morbidity, mortality and the costs involved in the treatment of infections, especially in human medicine. Currently, the dissemination of antibiotic resistances among bacteria and the struggle to develop new antimicrobial agents are compromising the effectiveness of antibiotic therapy (Cars & Nordberg, 2004). It is of great importance to understand which are the molecular mechanisms implied in the antibiotic resistance, in order to define new strategies and more efficient therapeutic approaches to infections, preventing the acquisition of new threatening antimicrobial resistances.

According to Alanis (2005), antimicrobial resistance could be natural/intrinsic, due to a spontaneous gene mutation, or acquired, as a result of the presence of antimicrobial agents and their selective pressure exerted on the bacteria. Antimicrobial resistance occurs at the genetic level. According to Neu (1992), bacteria become resistant due to the occurrence of three possible events: chromosomal mutation, introduction of new genetic information, whether by inductive expression of a latent chromosomal gene, and finally the exchange of genetic material between microorganisms. In order to understand the acquisition of these resistances, it is relevant to briefly describethe most common mechanisms of genetic transfer. According to Alanis (2005), these are conjugation, transformation and transduction, as seen in figure 8.

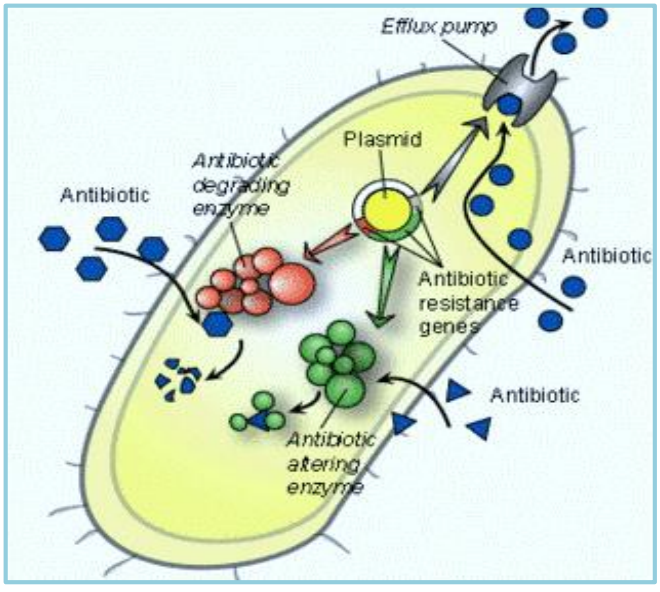
Figure 8 – Mechanisms of horizontal resistance gene transfer, adapted from Wang, J. (2006)



Conjugation is mediated by the bacteria plasmids, through a *pilus,* which is a structure responsible for the transferal of genetic information between bacteria. The result is the acquisition of extrachromosomal DNA. Transformation occurs with the passage of free DNA from dead bacteria to near receiving bacteria, and therefore its incorporation into the genetic code of the receiving bacteria (Neu, 1992). Transduction requires the presence of a vector (viruses) and occurs by infecting the receiving bacteria, at what time the bacterial resistance gene is introduced. Once the genetic information responsible for antimicrobial resistance is inside the bacteria, the expression of the resistance gene might interfere with the efficiency of the antimicrobial agent. This expression depends on the biological mechanisms of resistance, towards the antibiotics (as shown in figure 9). The main mechanism of resistance is the production of enzymes (e.g. β-lactamases), which degrade or modify the antibiotic structure. Antibiotic active efflux is another mechanism responsible for decreasing the antibiotic concentration inside the cell. Finally, the receptor modification interferes with the connexion between the cell and the molecules of the antimicrobial agent. (Neu, 1992; Alanis, 2005)

Several factors have a role in the acquisition of antimicrobial resistances. The selective pressure exerted by the use of antibiotics in agriculture and food, as well as the excessive and/or inadequate use of these agents in patients, is accountable for most of the resistances that currently exist (Alanis, 2005).

Figure 9 – Biological resistance mechanisms of bacteria, adapted from Wang, J. (2006)



2.3.1. Parameters of Antimicrobial Resistance Evaluation

The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013) provides harmonised data on inhibition zone diameter distributions for individual organisms and antimicrobial agents, on its website. There are two parameters available to analyse the

inhibition zone diameters obtained in the disk diffusion method, the clinical zone diameter breakpoints and the epidemiological cut-offs.

2.3.1.1. Clinical zone diameter breakpoints

This parameter is used to evaluate the susceptibility of the microorganisms to the tested antimicrobial agents, applied to clinical practice.

Clinically Susceptible (S ≥ x mm) - this is the classification applied to a microorganism that is inhibited by a determined level of an antimicrobial agent. A susceptible microorganism exhibits an inhibition zone diameter equal or larger than the susceptibility reference diameter, given by EUCAST. *In vitro* susceptibility is associated to a high likelihood of therapeutic success.

Clinically Intermediate (x < I ≤ y mm) – this is the classification applied to a microorganism which inhibition by a determined level of an antimicrobial agent is uncertain. An intermediate microorganism exhibits an inhibition zone diameter smaller than the susceptibility reference diameter given by EUCAST, but larger than the resistant reference diameter. The therapeutic success of the antimicrobial agent depends on the drug dosage and on concentrations achieved in the body tissues where the infection is located. This classification indicates that the agent is not a reliable therapeutic choice and an alternative antimicrobial agent must be tested.

Clinically Resistant (R < y mm) is the classification applied to a microorganism that is not inhibited by a determined level of an antimicrobial agent. This is associated with a high likelihood of therapeutic failure. A resistant microorganism exhibits an inhibition zone diameter equal or smaller than the resistant diameter.

2.3.1.2. Epidemiological cut-off values (ECOFF)

This parameter is useful to distinguish between wild type and non-wild type microorganisms. However, the epidemiological resistance does not have a clinical value, as the success of antimicrobial therapy, in both classifications of microorganisms, requires using clinical parameters.

Wild type applies to a bacterial isolate which does not possess acquired and mutational resistance mechanisms to the tested antimicrobial agent.

Non-wild type applies to a bacterial isolate that relies on acquired or mutational resistance mechanisms to the tested antimicrobial agent.

Multidrug resistant bacteria (MDR) is the classification applied to strains which possess acquired resistance to, at least, one antimicrobial agent from three or more antibiotic families

(Magiorakos *et al.*, 2012). A wide range of antimicrobial agents ought to be tested, in order to evaluate if a studied strain is MDR.

2.3.2. Resistance to β-lactams

The β-lactamases constitute a heterogeneous group of enzymes that are responsible for the most common mechanism of resistance to β-lactams. Their characteristic ring is the target of β-lactamases, which disrupt this structure rendering the molecules inefficient against bacteria. Over 890 distinct β-lactamases have been identified so far, and are assigned to different groups according to the enzymes' amino acid sequences or their activity against β-lactams (Bush, 2010; Bush & Jacoby, 2010).

Despite having a similar topology, these globular proteins amino acid sequences differ from each other. The Ambler molecular classification scheme and the Bush-Jacoby-Medeiros functional classification system are used to classify these enzymes (see Table 1). The former divides them into four groups: A, C and D include serine β-lactamases and B comprises metallo-β-Lactamases. As for Bush-Jacoby-Medeiros classification, it is based on the β-lactamases' functional features, and divides them into four groups according to their substrate and inhibitor profiles. Group 1 contains cephalosporinases not inhibited by clavulanic acid; group 2 - penicillinases, cephalosporinases, and broad-spectrum β-lactamases, inhibited by β-lactamase inhibitors; group 3 comprises metallo-β-lactamases that are capable of hydrolysing penicillins, cephalosporins, and carbapenems, but are poorly inhibited by almost all β-lactam-containing molecules; group 4 includes penicillinases that are poorly inhibited by clavulanic acid (Paterson & Bonomo, 2005; Perez, Endimiani, Hujer & Bonomo, 2007).

Table 1 – β-lactamases classification according to Bush-Jacoby-Medeiros and Ambler scheme. Adapted from The continuous challenge of ESBLs, Perez et.al 2007

Bush-Jacoby-Medeiros system	Major subgroups	Ambler System	Main attributes
Group 1 cephalosporinases	–	C (cephalosporinases)	Usually chromosomal; Resistance to all β-lactams except carbapenems
Group 2 penicillinases (clavulanic acid susceptible)	2a	A (serine β-lactamases)	Staphylococcal penicillinases
	2b	A	Broad-spectrum – TEM-1, TEM-2, SHV-1
	2be	A	Extended-spectrum – TEM-3–160, SHV-2–101
	2br	A	Inhibitor resistant TEM (IRT)
	2c	A	Carbenicillin-hydrolyzing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2d	D(oxacillin-hydrolyzing)	Cloxacillin-hydrolyzing (OXA)
Group 3 metallo-b-lactamase	3a	B (metalloenzymes)	Zinc-dependent carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

2.3.2.1. Cephalosporinases or AmpC β-lactamases

The AmpC enzymes are among the most abundant β-lactamases (Bush, 2010). Bacterial strains overexpressing these β-lactamases are usually resistant to all β-lactam agents,

including their association with β-lactamases inhibitors, except for cefepime, cefpirome and the carbapenems (European Food Safety Association - EFSA, 2011). This overexpression may occur as a result of the chromosomal gene deregulation (derepressed mutants) or the acquisition of this gene by a plasmid – plasmid-mediated AmpC β-lactamases, such as CMY, DHA, MOX, among others (Pérez-Pérez & Hanson, 2002; Bush, 2010). Plasmid-mediated *amp*C genes are derived from the chromosomal genes and have been detected in several genera of the Enterobactereacea family, essentially in *K. pneumoniae* and *E.coli* nosocomial isolates (Pérez-Pérez & Hanson, 2002). It is assumed that the production of the AmpC β-lactamases is associated with inappropriate use of cephalosporins in the past (Bakthavatchalu, Shakthivel & Mishra, 2013).

The co-production of ESBL and AmpC β-lactamases, by some resistant strains, is an emergent topic. Identifying strains harbouring multiple β-lactamases-coding genes is of great importance to choose effective antimicrobial therapy. However, it is challenging in that there is not a specific method to identify both ESBLs and AmpC (Bakthavatchalu, Shakthivel & Mishra, 2013). In table 2 is resumed the hydrolysis profile of these β-lactamases.

Table 2 - Hydrolysis profile of ESBLs and AmpCs, adapted from "Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals"; EFSA, 2011

ß-lactamase	Hydrolysis profile				Inhibited by clavulanic acid
	CAZ/CTX	FOX	FEP	IPM	
ESBL	+	-	+	-	Yes
AmpC	+	+	-	-	No

Abbreviations: CAZ – Ceftazidime, CTX – Cefotaxime, FOX – Cefoxitin, FEP – Cefepime, IPM – Imipenem

2.3.2.2. Penicillinases

The SHV-1 and the TEM-1 enzymes are found at high prevalence in several genera of the Enterobacteriaceae family. These were first described prior to the introduction of broad-spectrum cephalosporins, such as cefotaxime and ceftazidime and are considered Narrow spectrum β-lactamases (NSBLs). The association of β-lactamases inhibitors with β-lactams is very effective in infections caused by microorganisms producing a single penicillinase (Bush, 2010).

SHV

The SHV-type β-lactamases have been detected in most genera of Enterobacteriaceae and also most recently in *Pseudomonas aeruginosa* and *Acinetobacter* (Paterson & Bonomo, 2005).

Recent extended-spectrum SHV are possibly resultant from the mutation of narrow-spectrum chromosomal SHV-1 or SHV-2 β-lactamases, originated from the chromosome of *K.*

pneumoniae. In Europe, mainly SHV-12 and SHV-2 ESBLs have been detected in food-producing animals' isolates (EFSA, 2011).

TEM

The TEM-type β-lactamases so far identified are expressed essentially by *K. pneumoniae* and *E. coli*. They are derivatives of TEM-1 and TEM-2 variants which are able to hydrolyse ampicillin, carbenicillin, oxacillin and cephalothin, being inhibited by clavulanic acid. More than 100 TEM variants have been described, being most of them considered extended-spectrum β-lactamases (ESBLs). The variants TEM-1, TEM-2 and TEM-13 are considered narrow spectrum β-lactamases (Paterson & Bonomo, 2005).

2.3.2.3. Oxacillinases

Most of these enzymes are not able to hydrolyse cephalosporins to a significant degree, therefore most of OXA variants are considered narrow-spectrum β-lactamases (NSBLs). OXA-1 is the most common OXA enzyme and has been found in *E. coli* isolates (Patterson & Bonomo, 2005).

2.3.2.4. Extended-Spectrum β-Lactamases (ESBLs)

Extended Spectrum β-lactamases are produced by most genera of Enterobactereacea. These enzymes are originated from the NSBLs SHV-1 and TEM-1. Their appearance is the result of the introduction of cephalosporins that are stable in the presence of β-lactamases and aztreonam. ESBLs are associated to the outbreak of cephalosporin-resistant infections caused by *E. coli* and *K. pneumoniae* (Paterson & Bonomo, 2005). The genes coding for these enzymes are mostly plasmid-encoded and therefore transferable, and are currently spread worldwide (Bush, 2010).

In conclusion, ESBL-producing bacterial strains possess antimicrobial resistance to penicillins (e.g, ampicillin and piperacillin), 1st, 2nd, 3rd and 4th generation cephalosporins and aztreonam, but not to carbapenems or cephamycins (e.g. cefoxitin) (Paterson & Bonomo, 2005). The combination of β-lactams (amoxicillin) and β-lactamase inhibitors, such as clavulanic acid, enhances these compounds efficiency and inactivates ESBLs, as exemplified in table 2.

Several ESBLs have been identified up until this moment and were originated by mutations of NSBLs. This could be explained by the selective pressure exerted by the introduction of third-generation cephalosporins, in the past. Most of the extended spectrum enzymes belong to the molecular class A (Ambler classification scheme) with the exception of OXA-type ESBLs. The TEM-type are derivatives of TEM-1 and TEM-2. As for SHV ESBLs, these enzymes are derived from SHV-1 and are designated SHV-2 (Paterson & Bonomo, 2005). CTX-M-type are explained in detail below.

2.3.2.4.1. CTX-M

This is the most prevalent ESBL and is currently disseminated worldwide (Paterson & Bonomo, 2005). CTX-M-type ESBLs became the most frequent enzymes produced by clinical isolates of resistant bacteria, and have a major significance in nosocomial infections. Therefore a growing effort to detect and isolate infected individuals is being made, in order to prevent further spreading of CTX-M-type producing bacterial strains (Paterson & Bonomo, 2005; Perez *et al.*, 2007). *Escherichia coli* isolates expressing these ESBLs have been frequently obtained from human patients with urinary tract infections. The detection of CTX-M-type ESBLs is frequent in *K. pneumoniae, E. coli, Salmonella* spp. and *Enterobacter* spp. isolates (Perez *et al.*, 2007).

Most of these enzymes have similar hydrolysis profiles, namely cefotaxime and ceftriaxone, but not ceftazidime. However, there is a specific group with increased ceftazidime-hydrolysing activity. CTX-M-15 belongs to the latter group (Baraniak, Fiett, Hryniewicz, Nordmann & Gniadkowski, 2002).

Genes encoding CTX-M-15 have been found with a higher prevalence relevance in Italy, Portugal and France. Also it appears that food supply is a potential reservoir of resistant bacteria and genetic determinants -ESBL-producing bacteria, including strains harbouring $bla_{\text{CTX-M-15}}$, have been recovered from poultry (Tzouvelekis, Tzelepi, Tassios & Legakis, 2000; Baraniak *et al., 2002;* Paterson & Bonomo, 2005; Perez *et al.*, 2007).

2.3.2.4.2. Other β-Lactamases

Structurally similar to CTX-M, Toho-1 and Toho-2 are β-lactamases capable of hydrolysing the ring of the β-lactam antibiotics. These enzymes exhibit a greater activity against cefotaxime than ceftazidime (Paterson & Bonomo, 2005).

PER-1 was detected in *Pseudomonas aeruginosa, Salmonella enterica* serovar *Typhimurium* and *Acinetobacter* spp. isolates. It hydrolyses penicillins and cephalosporins, being inhibited by clavulanic acid. VEB-1 is also a plasmid mediated β-lactamase which confers high-level resistance to ceftazidime, cefotaxime and aztreonam (Paterson & Bonomo, 2005).

2.3.2.5. *mecA*

In 1992, Neu mentioned that 95% of the *Staphylococcus aureus* worldwide were resistant to penicillin and ampicillin. Likewise, a study from Wielders, Fluit, Brisse, Verhoef & Schmitz (2002) identified the *mecA* gene in 95% of the total isolated strains in which a phenotype of methicillin resistance has been detected. Methicillin was synthetized by the pharmaceutical industry to eliminate these resistant strains, however the resistance evolved from penicillin and ampicillin to methicillin as well. Methicillin-resistant *S. aureus* (MRSA) are able to inactivate all β-lactams antibiotics due to the presence of *mec*A gene, which encodes the

low-affinity penicillin-binding protein PBP 2A (MRSA are resistant not only to penicillins but also to cephalosporins, carbapenems and monobactams). Many MRSA are resistant to others antibiotics such as erythromycin, fusidic acid, tetracycline, minocycline, streptomycin, spectinomycin and sulfonamides, due to transposition and site specific integration in these strains chromosome. Coagulase-negative staphylococci such as *S. haemolyticus* and *S. hominis* are likewise β-lactamase producers and many possess the PBP2a. Actually, these staphylococci act as genes reservoirs, and play a role in their transmission to *S. aureus.*
Staphylococci are usually resistant to aminoglycosides. This resistance was originated by the synergistic association of aminoglycosides along antistaphylococcal penicillins in the past. (Neu, 1992)

According to Monecke *et al.* (2013) *mecC* is a recently described gene in *S. aureus* and coagulase-negative staphylococci and it is considered a *mecA* homolog. This gene is responsible for antimicrobial resistance to β-lactam antibiotics and raises concern over possible development of new methicillin resistance mechanisms.

2.3.3. Resistance to aminoglycosides

Aminoglycosides are subjected to enzymatic inactivation by acetyltransferases (AAC), nucleotidyltransferases (ANT) and phosphotransferases (APH). A selection of these enzymes was made in order to include a brief description of the most relevant for the present study. The aminoglycoside N-acetyltransferases constitute the AACs superfamily which is a large group of enzymes that have in common the ability of catalysing the acetylation of a primary amine from aminoglycosides antibiotics. According to its acetyl group position, these proteins are assigned in different subgroups: AAC(1'), AAC(3'), AAC(2′), or AAC(6′) (Ramirez, Nikolaidis & Tolmasky, 2013).
The AAC(3)-I can be found in several genera from Enterobactereacea family and provides bacterial resistance to gentamycin and fortimycin. Two distinct DNA sequences have been identified, aac(3)-Ia and aac(3)-Ib. The AAC(3)-II confers resistance to gentamicin, tobramycin, dibekacin, netilmicin, among other aminoglycosides (Shaw, Rather, Hare & Miller, 1993).
As for the AAC(6′) this subgroup comprises more enzymes than any other AAC subgroup. According to Ramirez *et al.* (2013) about 40 different proteins have been identified in both Gram-negative and Gram-positive isolates. AAC(6′)-Ib enzymes can be assigned into two distinct groups according to their antimicrobial resistance profile - the AAC(6')-I enzymes modify amikacin and gentamicin, while AAC(6')-II enzymes interfere with the antimicrobial activity of gentamicin, but not amikacin (Shaw *et al.*, 1993).

3. Bacterial species

3.1. Enteric pathogens

3.1.1. *Escherichia coli*

Included in the Enterobactereacea family, *E. coli* is an aerobic medium size, Gram negative and oxidase-negative, rod shaped organism, that can be motile (petrichious flagella) or non-motile (Prescott et al, 2002; Quinn, Carter, Markey & Carter, 2002). Its optimum growth occurs at the temperature of 37ºC.

This bacterial species is a commensal microorganism inhabitant of the colon of humans and other warm-blooded animals, being an excellent indicator of faecal contamination. (Quinn *et al.,* 2002).

Pathogenic strains are usually associated with septicaemias in calves and with respiratory tract diseases. *Escherichia coli* strains are usually susceptible to amoxicillin, ampicillin, apramycin, chloramphenicol, furazolidone, kanamycin, spectinomycin, streptomycin, sulfonamides, tetracycline and trimethoprim (Bruner, Timoney & Hagan, 1988).

3.1.2. *Klebsiella oxytoca*

This facultative anaerobe is a Gram-negative, oxidase negative and non-motile rod. Also an intestinal commensal, *K. oxytoca* has the ability to acquire multiple antibiotic resistance. It is known as an opportunistic agent causing nosocomial infections in veterinary hospitals and this event is usually aggravated by the selective pressure caused by antibiotic's therapy, in these environments (Bruner *et al.*, 1988).

A study from Stock and Wiedemann (2001) from a total of 221 isolated strains of *Klebsiella* spp. was concluded that all strains were naturally resistant or intermediate to amoxicillin, ticarcillin among other agents to which other genera of Enterobacteriaceae are likewise intrinsically resistant. These strains are susceptible to some penicillins, all cephalosporins, aminoglycosides, quinolones, tetracyclines, trimethoprim, cotrimoxazole, chloramphenicol and nitrofurantoin.

3.1.3. *Morganella morganii*

This Enterobactereacea is a Gram-negative anaerobic rod, representing the only member of its genus. Although this species is often found as a commensal microorganism present in faeces and intestines of all kinds of mammals, it is an opportunistic agent. *Morganella morganii* is known, mostly in human medicine, for being the cause of opportunistic infections, both in the respiratory and urinary tract (Chen *et al.*, 2012).

This species has intrinsic resistance to β-lactams in general, such as oxacillin, ampicillin, and amoxicillin, 1G and 2G cephalosporins and to macrolides. (Bruner *et al.*, 1988, Nakazawa, *et al.*, 2013).

3.2. Staphylococci

These Gram-positive cocci are facultative anaerobes, oxidase-negative, rod shaped bacteria which occur in grapelike clusters (Bruner *et al.*, 1988; Prescott *et al.*, 2002).

Most pathogenic staphylococci species are usually coagulase-positive, which is the case of *Staphylococcus aureus*, while commensal species are coagulase-negative (Quinn *et al.,* 2002). As mentioned before*, S. aureus* isolation has been described in cetaceans, namely from the bottlenose dolphin respiratory tract and skin (Quinn *et al.*, 2002). *S. delphini* isolates*,* also coagulase-positive staphylococci, were recovered from purulent supurative lesion on the skin of bottlenose dolphins (Higgins, 2000).

These facultative anaerobes are often found in nasal secretions, due to their ability to grow under conditions of high osmotic pressure. It is known that *S. aureus* is often found in marine mammals, but its origin is uncertain. Two hypothesis are considered, whether these strains co-evolved with the marine mammals species and therefore are host species specific, or are originally from other host species (van Elk, Boelens, van Belkum, Foster, & Kuiken, 2012).

3.3. *Enterococcus faecalis*

These Gram positive cocci are facultative anaerobes, oxidase and catalase-negative. (Quinn *et al.,* 2002). *Enterococcus faecalis* with high antimicrobial resistance are a concern in that these strains are widespread and assume an important role in hospitals, causing nosocomial infections in humans. Enterococci produce β-lactamases similarly to Staphylococci, aminoglycoside-inactivating enzyme and may have high-level resistance mechanisms to glycopeptides such as vancomycin and teicoplanin. Vancomycin resistance was probably the result of an increased use of this agent in human medicine in the past, in order to fight MRSA infections that were becoming a growing concern (Neu, 1992).

III. Materials and Methods

1. Confirmation and characterization of the bacterial species

The bacterial isolates from this study were obtained, in 2011, as part of a previous study (Fernandes, 2012), through aseptic collection of blowhole exudate, performed by the trainers in cooperation with the veterinarian. The method used to collect the samples included the disinfection of the hands and the skin surrounding the blowhole of the dolphins with sterilized gauze soaked in chlorexidine. After a first wasted expiration performed at the request of the trainer, the area was disinfected again and the *sputum* collection was carried out, through three expirations directly into sterilized *petri* plates. According to Avallos-Télez *et al.* (2010), the microbiological sampling must be performed with no water contamination, with an adequate disinfection of the blowhole with sterilized gauze, and finally the sample should be collected into a sterile bag or container of broader size, approximately 2 cm wider than the blowhole diameter. As for the temporary storage of the biological samples, these should be maintained refrigerated at 4ºC, for a maximum period of 24 hours. After the identification of the bacterial species recovered from the *sputum,* the isolates were stored at the temperature of -20ºC for approximately two years, at the microbiology laboratory of the *Universidade de Trás-os-Montes e Alto Douro*.

Confirmation of the bacterial species from the representative samples

In order to confirm the bacterial species from the selected representative sample, and the purity of the colonies, the selected strains were inoculated and identified through different techniques. These were the BBL™ Crystal™ Gram Positive ID system (BD™, USA), API™ 20E and 20NE (bioMérieux™, France), and were performed as instructed by the manufacturer. The Polymerase Chain Reaction technique was also used to confirm the *E. coli* strains and to determine the staphylococci species.

2. DNA Extraction

Two different DNA extraction protocols were used in this study, adapted to the *E. coli* and *Staphylococcus* spp. species. This technique is necessary for the execution of the PCR technique, in which bacterial DNA is used to analyse genotypic characteristics of the studied bacterial strains.

2.1. DNA extraction of the staphylococci

This technique uses a disposable inoculation loop of the bacteria isolates immersed into a centrifuge tube with phosphate buffer saline (PBS). The next step is the centrifugation

followed by the removal of the supernatants and the re-suspension of the pellet in Tris-EDTA, which is finally boiled for about 10 minutes. After removing the tubes from the water, they must be placed in contact with ice for a minute. Afterwards, the DNAs are diluted and adequately identified and stored at -20º C.

2.2. DNA extraction of *E. coli*

This technique consists in collecting a significant amount of each colony and suspending it in sterile miliQ water. The following step is the centrifugation, and then the supernatant is wasted and the pellet re-suspended again in miliQ water. The ressuspended pellets are boiled for about 15 minutes. After this step, centrifugation must be completed. The supernatant is then removed and placed into new tubes, identified and stored at -20º C.

3. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was the technique chosen to proceed to the amplification of bacterial genes, thus obtaining further information on genotypic characteristics of the strains presenting antimicrobial resistances. This technique was using the PCR thermocycler (Eppendorf™, Hamburg, Germany). Detection of the respective PCR products was achieved by one dimensional electrophoresis, which is the technique used for most routine protein and nucleic acid separations. The gel used was agarose gel.

The list of the primers used in the different PCR performed, is given in the table 8, present in the attachments of the dissertation.

3.1. PCR for the staphylococci strains

mecA

The positive control used in order to perform the PCR was the strain ST80 MRSA PVL+. This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water, 10x PCR buffer (as instructed by the manufacturer), 1.5 mM of $MgCl_2$ (25 mM), 0.2 mM of dNTPs (25 mM), and the following primers – mecA-1, mecA-2, nuc-1, nuc-2, 16S-1, 16S-2 (see Table 8s), 0.26 of DMSO (5%) and, finally, 0.5 µl of NZYtaq. Thermal cycling reactions consisted of an initial denaturation (7 minutes at 94˚ C) followed by 30 cycles of annealing (5 minutes at 61˚ C), elongation (1 minute at 72˚ C) and denaturation (1 minute at 94˚ C), with a repetition of annealing (5 minutes at 61˚ C) and a final elongation (5 min at 72˚ C).

mecC

The positive control used in order to perform the PCR was the strain CGA 251.This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water,

10x PCR buffer (as instructed by the manufacturer), 0.5 mM of $MgCl_2$ (25 mM), 0.2 mM of dNTPs (25 mM), and the following primers – mecLGA251f and mecLGA251r (see Table 8) and, finally, 0.5 µl of Dream Taq. Thermal cycling reactions consisted of an initial denaturation (2minutes at 95˚ C) followed by 30 cycles of denaturation (30 s at 95˚ C), annealing (30 s at 56˚ C) and elongation (1 minute at 72˚ C), with a single final elongation (2 minutes at 72˚ C).

Identification of the bacterial species *Staphylococcus delphini*

There was not a strain from this bacterial species available, therefore no positive control was used in this PCR. This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water, 10x PCR buffer (as instructed by the manufacturer), 1 mM of $MgCl_2$ (25 mM), 0.2 mM of dNTPs (25 mM), and the following primers – dea-F, dea-R, deb-F, deb-R4 (see Table 8) and, finally, 0.5 µl of Dream Taq. Thermal cycling reactions consisted of an initial denaturation (2minutes at 95˚ C) followed by 30 cycles of denaturation (30 s at 95˚ C), annealing (30 s at 56˚ C) and elongation (1 minute at 72˚ C), with a single final elongation (2 minutes at 72˚ C).

3.2. PCR for the *E. coli* strains

3.2.1. β-lactamases

TEM, SHV and OXA-1 genes

The positive controls used in order to perform the PCR were the strains *E. coli* 5825/04 for OXA/TEM and *E. coli* K12 for SHV-5. This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water, 10x PCR NZYtech buffer (as instructed by the manufacturer), 3 mM of $MgCl_2$ (25 mM), 0.25mM of dNTPs (25 mM), and the following primers – P1, P2, shvf1, shvr, AmpCfor634, AmpCrev634, Oxa1f and Oxa1r (see Table 8), 0.26 µl of DMSO (5%) and, finally, 0.3 µl of NZYTaq. Thermal cycling reactions consisted of an initial denaturation (7 minutes at 94˚ C) followed by 30 cycles of annealing (5 minutes at 61˚C), elongation (1 minute at 72˚C) and denaturation (1 minute at 94˚C), with a repetition of annealing (5 minutes at 61˚ C) and a final elongation (5 min at 72˚C) (Pomba *et al.*, 2006).

CTX-M genes

The positive control used in order to perform the PCR was the strain *E. coli* 5825/04.This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water, 10x PCR buffer (as instructed by the manufacturer), 1.5 mM of $MgCl_2$ (25 mM), 0.2 mM of dNTPs (25 mM), and the following primers – ctx-m-1-seqf and ctx-m-1-seqr (see

Table 8), 0.26 of DMSO (5%) and, finally, 0.5 µl of NZYtaq. Thermal cycling reactions consisted of an initial denaturation (3 minutes at 94˚ C) followed by 30 cycles of denaturation (1 minute at 94˚ C), annealing (30 s at 59˚ C) and elongation (1 minute at 72˚ C), with a single final elongation (7 minutes at 72˚ C). (Pomba *et al.*, 2006).

AmpC genes

The positive controls used in order to perform the PCR were the strains E.coli J53PMG144, E.coli J53PMG251, E.coli J53PMG252, Proteus 1089/07, Kebsiella Nu2936 and Klebsiella 96D. This PCR was performed in a final volume of 50 µl containing 2.5 µl of the DNA template, sterile MiliQ water, 5x PCR buffer NZYtech (as instructed by the manufacturer), 1.5 mM of $MgCl_2$ (25 mM), 0.25 mM of dNTPs (25 mM), and the following primers – MOXMF, MOXMR, CITMF, CITMR, DHAMF, DHAMR, ACCMF, ACCMR, EBCMF, EBCMR, FOXMF and FOXMR (see Table 8), 0.26 of DMSO (5%) and, finally, 0.5 µl of NZYtaq. Thermal cycling reactions consisted of an initial denaturation (3 minutes at 94˚ C) followed by 30 cycles of denaturation (30 seconds at 94˚ C), annealing (30 s at 65˚ C) and elongation (1 minute at 72˚ C), with a single final elongation (7 minutes at 72˚ C).

3.2.2. Mechanisms of aminoglycoside resistance

aaC (6') - Ibr PCR

The positive control used in order to perform the PCR was the strain 5825/04. This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water, 10x PCR buffer (as instructed by the manufacturer), 0.75 mM of $MgCl_2$ (25 mM), 0.4 mM of dNTPs (25 mM), and the following primers – AAC6Ibf3, AAC6Ibr3 (see Table 8), 0.26 of DMSO (5%) and, finally, 0.3 µl of Dream Taq. Thermal cycling reactions consisted of an initial denaturation (7 minutes at 94˚ C) followed by 30 cycles of annealing (5 minutes at 61˚ C), elongation (1 minute at 72˚ C) and denaturation (1 minute at 94˚ C), with a repetition of annealing (5 minutes at 61˚ C) and a final elongation (5 min at 72˚ C).

aaC (3') – IV PCR

The positive control used in order to perform the PCR was a positive strain, previously detected in an on-going project at the laboratory. This PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, sterile MiliQ water, 10x PCR buffer (as instructed by the manufacturer) 1.5 mM of $MgCl_2$ (25 mM), 0.25 mM of dNTPs (25 mM), and the following primers – AacC4f and AacC4r (see Table 8), 0.26 of DMSO (5%) and, finally, 0.4 µl of NZYTaq. Thermal cycling reactions consisted of an initial denaturation (5 minutes at 94˚ C) followed by 30 cycles of denaturation (1 minute at 94˚ C), annealing (1 minute at 55˚ C), elongation (1 minute at 72˚ C) and a final elongation (10 min at 72˚ C).

4. Antimicrobial Susceptibility Test

4.1. Methicillin resistance test

A selective medium, Brilliance^{TM} MRSA 2 agar (Oxoid®, United Kingdom), was used to detect methicillin resistant strains by plating the suspected staphylococci colonies. Only the colonies expressing resistance to methicillin are able to grow in this agar, therefore, it is highly likely that any colonies present after the incubation period (18-24 hours, 37ºC) possess the *mec*A *or mec*C gene. This assumption should be confirmed afterwards through PCR.

4.2. Disk diffusion method

Antimicrobial susceptibility testing was performed by the disk diffusion method. The first step of this technique consists in inoculating a single and well isolated colony into 5mL of sterile water solution with NaCl (0.85%), using a sterile swab (0.5 MacFarland standard). With the same swab, this solution was inoculated in Müeller-Hinton agar plates, filling the whole area. Antimicrobial disks (see table 9) were then placed on the surface of the agar and the plates were incubated for an 18-24 hour period at 37ºC. The antimicrobial susceptibility of the strains was evaluated through the measurement of the transparent halos formed around the disks. The zone diameter was then compared with the breakpoints established for each antimicrobial agent and bacterial species.

In this study, EUCAST's Epidemiological cut-offs (when available) and CLSI clinical breakpoints were used to analyse the antimicrobial resistance data obtained by this method.

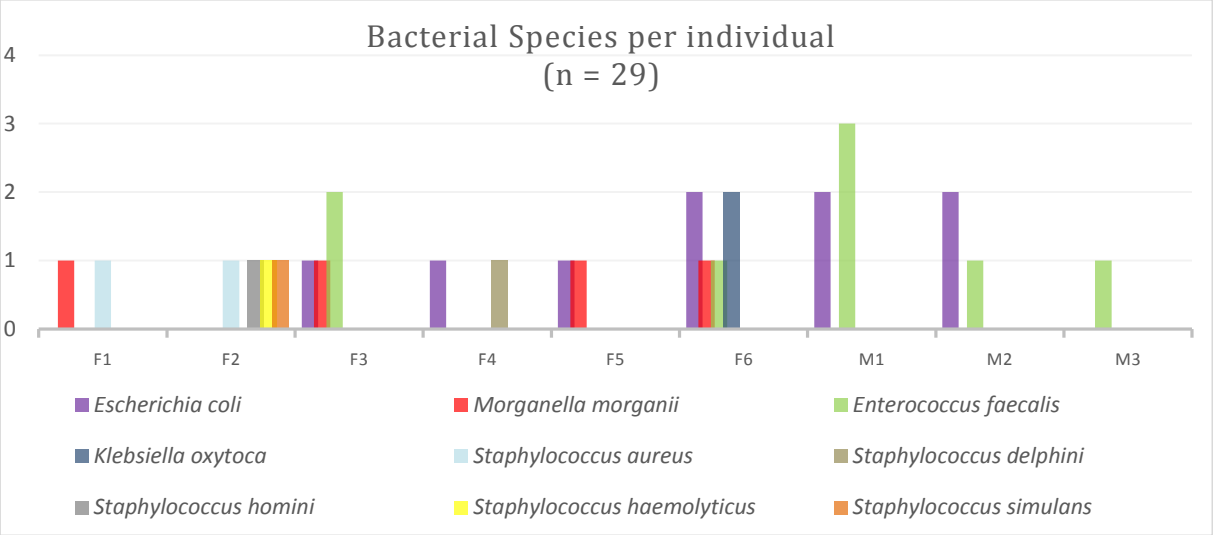
The selection of the antimicrobial disks is present in the attachments, in table 9.

IV. Results and Discussion

1. Confirmation of the bacterial species

In a previous study performed by Fernandes (2012), several bacterial species were recovered from 9 bottlenose dolphins, sampled for blowhole exudate. A representative sample of 29 isolates was selected, in order to study the antimicrobial resistance of these isolates. Chart 1 resumes these findings.

Chart 1– Graphic representation of the bacterial species isolated per individual



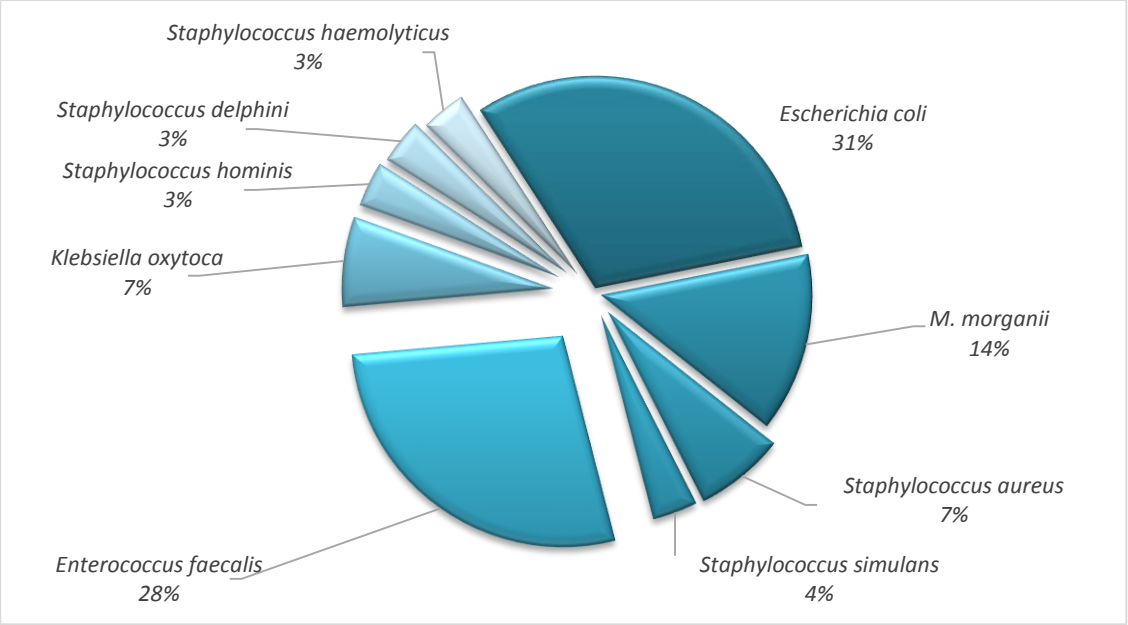
The bacterial species recovered from the females were the following - From F1 individual, one isolate of *Morganella morganii* and one of *Staphylococcus aureus*. From F2, one isolate of *S. aureus, S. hominis, S. haemolyticus* and *S. simulans*. From F3, one isolate of *M. morganii* and *E. coli* and two isolates of *Enterococcus faecalis*. From F4, one *E. coli* and one *S. delphini.* From F5, one isolate of *M. morganii* and *E. coli*. From F6 two isolates of *E. coli* and *Klebsiella oxytoca,* and one isolate of *M. morganii* and *E. faecalis*.

As for the isolates recovered from the males - from M1, four *E. faecalis* isolates and two of *E. coli* were identified. From M2, two isolates of both *E. faecalis* and *E. coli.* From M3, two isolates of *E. faecalis.*

In chart 2 is demonstrated the distribution of the recovered bacterial species.

The most frequently isolated bacterial species were *E. coli* (31%), followed by *E. faecalis* (28%), *M. morganii* (13%), *K. oxytoca* (7%) and with less significance five different staphylococci: *S. aureus* (7%), *S. delphini* (3%), *S. simulans* (3%), *S. hominis* (3%) and *S. haemolyticus* (3%).

Chart 2 - Graphic representation of the distribution of the recovered bacterial species



Buck *et al.* (2006) performed a study that aimed to identify aerobic microorganisms in free-range dolphins inhabiting the coastal Gulf of Mexico and Atlantic Ocean waters. The bacterial species recovered from the blowhole and faecal samples, in healthy individuals, were *Vibrios* spp.*, E. coli, E. tarda, Citrobacter freundii, Flavobacterium meningosepticum, Hafnia alvei, Klebsiella pneumoniae, Morganella morganii, Pasteurella multocida, Proteus* spp., *Providencia* spp.*, Serratia* spp.*, Yersinia enterocolitica,* some unidentified *Pseudomonas* spp., *Staphylococcus* spp. and *Streptococcus/Enterococcus* spp. (Buck *et al.*, 2006). Here, Buck *et al.* (2006) considered that some of the enteric bacteria found, namely staphylococci and streptococci/enterococci, could be the result of human contamination during the sampling procedures.

From 1993 to 2000, a similar study was performed in a group of 15 cetaceans under human care, in Hong Kong. Here, Chan, Mukherjee, Kinoshita & Yuen (2001) described the identification of *Vibrio alginolyticus, Proteus mirabilis, Shewanella putrefaciens, M. morganii, S. aureus* and *P. aeruginosa*.

The studies from Buck *et al.* (2006) and Chan *et al.* (2001), allow a comparison between cetaceans under human care and from the wild, since some bacterial species were recovered in both groups of animals – *Vibrios* spp., *Proteus* spp., *Pseudomonas* spp., *M. morganii* and *Staphylococcus* spp.

In the current study, the species *E. coli, E. faecalis*, *M. morganii*, *Klebsiella oxytoca*, *S. aureus*, *S. delphini*, *S. simulans*, *S. hominis* and *S. haemolyticus* were identified as part of the microbiota of the studied bottlenose dolphins. These findings are similar to previous studies from authors, such as Buck *et al.* (2006) and Chan *et al.* (2001), meaning that these microorganisms are normally present in the upper respiratory tract of the bottlenose dolphin.

The differences between the composition of microbiota from free-range and under human care dolphins are the result of the exposure of these animals to different bacterial species. A series of parameters interfere with the microbiota composition. For example, in the habitat of free range individuals, there is a wider variety of microorganisms, due to anthropogenic toxins originated from human pollution and industrial/agricultural effluents released in the ocean. (Varela *et al.*, 2007). Also, oceanographic physical properties, such as water temperature and currents, influence greatly the necessary conditions for bacterial growth (Tlaskalova-Hogenova *et al.*, 2004; Wells, 2009). The occurrence of biomagnification, in wild marine top-level predators, is associated to the presence of environmental contaminants in seawater, as well. Heavy metals and organohalogen compounds are magnified along the food chain and might interfere with the microbiota composition and the immunity of an individual (Blackburn, 2003; Tlaskalova-Hogenova *et al.*, 2004). Dolphins under human care can be fed with wild-caught fish and therefore this phenomenon might be observed in these individuals, as well.

Besides the mentioned parameters, some microorganisms might be introduced by humans and other vectors contacting with the dolphins.

Most of the microorganisms isolated from the respiratory tract of bottlenose dolphins with pneumonia, are often present, as well, in the URT of healthy individuals (Cusick & Bullock., 1973; Avalos-Téllez *et al.*, 2010; Venn-Watson *et al.*, 2011). This is a strong indicator that the onset and development of an infection, does not only depend on the bacterial species involved, but also on another risk factors, such as individual susceptibility, husbandry measures and environmental parameters (Wells, 2009). Some cetaceans, from zoological collections, were born in zoological/ oceanographic parks and have inhabited, since birth, a closed habitat, while others were born in the wild and taken under human care, afterwards. The normal microbiota composition is established through a balance between the environment and the individual, from the moment he was born (Tlaskalova-Hogenova *et al.*, 2004). Dolphins inhabiting pools are fed several times a day, and eliminate their excrements in the same water they live in. Despite the cleaning procedures, and chemical products used to eliminate organic matter, achieving a complete disinfection is a challenge. Chlorine and other antimicrobial components that are added to the water, might interfere with the normal microbiota composition and stability, causing the pathogens to proliferate and cause an infection (Avalos-Téllez, 2010). An adequate disinfection of the pools of oceanographic/ zoological parks demands the transference of the animals to other facilities, in order to carry out a sanitary break. However, logistically, this is a difficult procedure to carry out.

According to Avalos-Téllez *et al.* (2010), alterations in the host environment may interfere with the commensalism of the microbiota, leading to the development of an infection. Buck *et al.* (2006) suggested that, in comparison to healthy individuals, debilitated animals, such as

those found stranded, might have a narrower spectrum of microorganisms albeit in a larger quantity.

2. Antimicrobial resistance results

2.1. Enterobacteriaceae

Antimicrobial susceptibility was evaluated through disk diffusion performed in all bacterial species. Against the recovered isolates from the Enterobactereacea family, 27 antimicrobial agents were tested.

2.1.1. *Escherichia coli*

The antimicrobial susceptibility of the *E. coli* isolates was tested through the disk diffusion method and the resistances found were afterwards studied through PCR. Several resistance genes against β-lactams, including extended-spectrum β-lactamases, narrow-spectrum β-lactamases, AmpC β-lactamases coding genes and also aminoglycoside resistance genes were investigated. In table 3 are resumed the obtained resistance profiles. The clinical resistance results are shown in the first column, followed by the total of families to which the strains were resistant and in the second column are the antimicrobial agents to whom the strains were considered non-wild type. The parameters used are presented in table 10, as well as the strains' classification, according to the inhibition zone diameters observed.

Table 3 – Antimicrobial resistance results of the *Escherichia coli* isolates.

Strain	Clinical Resistances Profile	Epidemiological Resistances Profile	*Classification according to the EUCAST parameters* (in antibiotic families)	Harboured genes
Ec1	**Penicillins:** Aml^R **Cephalosporins:** $Cl^R Ctx^R$ **Tetracyclines**:Te^R **Quinolones**: $Cip^R Enr^R Lev^R Na^R$ **Aminoglycosides**:$Cn^R Tob^R$ **Sulfonamides:**$S_3{}^R$ **Trimethoprim**:$Sxt^R W^R$ Clinically intermediate: $K^I Mem^I$	$Aml^{NWT} Cl^{NWT} Ctx^{NWT} Fep^{NWT} Te^{NWT} Na^{NWT} Cip^{NWT} Lev^{NWT} Cn^{NWT} Tob^{NWT} Sxt^{NWT} W^{NWT} Mem^{NWT}$	Non wild type 7 Clinical resistance 7	bla_{TEM} $bla_{CTX\text{-}M\text{-}15}$
Ec2	**Penicillins:** Aml^R **Cephalosporins:** $Cl^R Kf^R Fep^R Cxm^R$ **Tetracyclines**:Te^R **Quinolones**: $Cip^R Enr^R Lev^R Na^R$ **Aminoglycosides**:$Cn^R Tob^R K^R$ **Chloramphenicol:** C^R **Trimethoprim**:$Sxt^R W^R$ Clinically intermediate: $S3^I Ert^I Mem^I$	$Aml^{NWT} Cl^{NWT} Fep^{NWT} Cxm^{NWT} Te^{NWT} Cip^{NWT} Lev^{NWT} Na^{NWT} Cn^{NWT} Tob^{NWT} W^{NWT} Mem^{NWT} Ipm^{NWT} Etp^{NWT}$	Non wild type 7 Clinical resistance 7	bla_{TEM} $bla_{CTX\text{-}M\text{-}32}$
Ec3	**Penicillins:** $Aml^R Amc^R$ **Cephalosporins:** $Cl^R Kf^R Fep^R Cxm^R Ctx^R Caz^R$ **Tetracyclines**:Te^R **Quinolones**: $Cip^R Enr^R Lev^R Na^R$ **Aminoglycosides**: $K^R Cn^R Tob^R$ **Carbapenems:** Mem^R Clinically intermediate: $Ert^I Fos^I W^I$	$Aml^{NWT} Amc^{NWT} Cl^{NWT} Fep^{NWT} Cxm^{NW} Ctx^{NWT} Caz^{NWT} Te^{NWT} Na^{NWT} Cip^{NWT} Lev^{NWT} Cn^{NWT} Tob^{NWT} W^{NWT} Mem^{NWT} Etp^{NWT}$	Non wild type 7 Clinical resistance 6	$bla_{OXA\text{-}1}$ $bla_{CTX\text{-}M\text{-}32}$ $bla_{DHA\text{-}1}$ *aac(6')-Ib*

Strain	Clinical Resistances Profile	Epidemiological Resistances Profile	*Classification according to the EUCAST parameters* (in antibiotic families)	Harboured genes
Ec4	**Penicillins:** Aml^R **Cephalosporins:** Cl^RCxm^R **Quinolones**: $Cip^REnr^RLev^RNa^R$ **Aminoglycosides**: K^R **Chloramphenicol:** C^R Clinically intermediate: $Kf^IMem^IS3^ITe^ITob^I$	$Aml^{NWT}Cl^{NWT}Fep^{NWT}Cxm^{NWT}Te^{NWT}$ $Na^{NWT}Lev^{NWT}Cip^{NWT}C^{NWT}W^{NWT}$ $Mem^{NWT}Etp^{NWT}$	Non wild type 7 Clinical resistance 5	bla_{TEM}
Ec5	**Penicillins:** Aml^R **Cephalosporins:** $Cl^RKf^RFep^RCxm^RCtx^RCaz^R$ **Quinolones**: $Cip^REnr^RLev^RNa^R$ **Aminoglycosides**:$Cn^RK^RTob^R$ **Carbapenems:** Mem^R **Fosfomycin:** Fos^R Clinically intermediate: Ert^IAk^I	$Aml^{NWT}Amc^{NWT}Cl^{NWT}Fep^{NWT}Ctx^{NWT}$ $Caz^{NWT}Cxm^{NWT}Te^{NWT}Na^{NWT}Lev^{NWT}$ $Cip^{NWT}Cn^{NWT}Tob^{NWT}Mem^{NWT}$ Etp^{NWT}	Non wild type 6 Clinical resistance 6	bla_{OXA_1} $bla_{CTX\text{-}M\text{-}15}$ *aac(6')-Ib*
Ec6	**Penicillins:** Aml^RAmc^R **Cephalosporins:** $Cl^RKf^RFep^RCxm^RCtx^RCaz^R$ **Quinolones**: $Cip^REnr^RLev^RNa^R$ **Aminoglycosides**:$Cn^RK^RTob^R$ **Fosfomycin:** Fos^R **Sulfonamides:**$S_3{}^R$ **Trimethoprim**:Sxt^RW^R Clinically intermediate: Ert^ITe^I	$Aml^{NWT}Amc^{NWT}Cl^{NWT}Fep^{NWT}Ctx^{NWT}$ $Caz^{NWT}Cxm^{NWT}Te^{NWT}Na^{NWT}Lev^{NWT}$ $Cip^{NWT}Cn^{NWT}Tob^{NWT}Sxt^{NWT}W^{NWT}$ $Mem^{NWT}Etp^{NWT}$	Non wild type 7 Clinical resistance 7	$bla_{OXA\text{-}1}$ $bla_{CTX\text{-}M\text{-}15}$ *aac(6')-Ib*
Ec7	**Penicillins:** Aml^RAmc^R **Cephalosporins:** Cl^RKf^R **Tetracyclines**:Te^R **Quinolones**: $Cip^REnr^RLev^RNa^R$ **Aminoglycosides**:Cn^RTob^R **Sulfonamides:**$S_3{}^R$ **Trimethoprim**:Sxt^RW^R Clinically intermediate: $Kf^IK^IMem^I$	$Aml^{NWT}Amc^{NWT}Cl^{NWT}Fep^{NWT}Te^{NWT}$ $Na^{NWT}Lev^{NWT}Cip^{NWT}Cn^{NWT}Tob^{NWT}$ $Sxt^{NWT}W^{NWT}Mem^{NWT}$	Non wild type 7 Clinical resistance 7	bla_{TEM}
Ec8	**Penicillins:** Aml^R **Cephalosporins:** $Cl^RKf^RCxm^R$ **Quinolones**: $Cip^REnr^RLev^RNa^R$ **Aminoglycosides**: Tob^R **Trimethoprim**: W^R Clinically intermediate: $K^ISxt^ITe^IMem^I$	$Aml^{NWT}Cl^{NWT}Cxm^{NWT}Te^{NWT}Na^{NWT}$ $Cip^{NWT}Lev^{NWT}Tob^{NWT}Sxt^{NWT}W^{NWT}$ $Mem^{NWT}Etp^{NWT}$	Non wild type 7 Clinical resistance 5	bla_{TEM}
Ec9	**Penicillins:** Aml^RAmc^R **Cephalosporins:** $Cl^RKf^RCxm^R$ **Tetracyclines**:Te^R **Quinolones**: $Cip^REnr^RLev^RNa^R$ **Aminoglycosides**: $Cn^RK^RTob^R$ **Chloramphenicol:** C^R **Trimethoprim**: Sxt^RW^R **Sulfonamides:**$S_3{}^R$ Clinically intermediate: Mem^I	$Aml^{NWT}Amc^{NWT}Cl^{NWT}Fep^{NWT}$ $Cxm^{NWT}Te^{NWT}Na^{NWT}Lev^{NWT}Cip^{NWT}$ $Cn^{NWT}Tob^{NWT}W^{NWT}Sxt^{NWT}C^{NWT}$ Mem^{NWT}	Non wild type 8 Clinical resistance 8	bla_{TEM}

Abbreviations: Ec – *Escherichia coli*. According to EUCAST Clinical Breakpoints: R – Resistant; I – intermediate susceptibility. According to EUCAST Epidemiological cut-offs: NWT – Non Wild Type.
Aml – Amoxicillin, Amc – Amoxicillin + Clavulanic Acid; Cl – Cephalexin; Kf – Cephalotin; Fep – Cefepime; Ctx – Cefotaxime; Fox – Cefoxitin, Caz – Ceftazidime; Cxm – Cefuroxime; Te – Tetracycline; Na – Nalidixic acid; Cip – Ciprofloxacin; Enr – Enrofloxacin; Lev – Levofloxacin;; C – Choramphenicol;; Etp – Ertapenem; Ffc – Florphenicol; Fos - Fosfomycin, Cn – Gentamycin; Ak – Amikacin; K – Kanamycin; Tob - Tobramycin; Mem – Meropenem: Ipm – Imipenem;; S_3 - Sulfonamides compounds;; Sxt – Sulfonamide+trimethoprim; W – Trimethoprim.

The inhibition zone diameters observed in the antimicrobial susceptibility testing were analysed according to the clinical breakpoints given by EUCAST (2013) and CLSI Vet S1-02 (2013), when the former were not available. The epidemiological breakpoints of EUCAST allowed the identification of non-wild type strains, i.e. microorganisms with acquired or mutational resistance mechanisms to the tested antimicrobial agents.
The interpretation of the antimicrobial susceptibility results suggested an overall resistance to the antimicrobial agents tested.

β-lactams

The analysis of table 3 leads to the conclusion that the majority of the *E. coli* isolates was resistant to β-lactam agents. Seven strains (from Ec 3 to Ec 9) demonstrated to be resistant to the amoxicillin-clavulanic acid association, with the exception of the strains Ec 1 and Ec 2, which growth was inhibited by this agent. As for the resistance to cephalosporins, all nine isolates were not inhibited by cephalotin and/or cephalexin, first generation agents. With the exception of Ec 1 and Ec 7, the other strains demonstrated resistance to cefuroxime (2nd G). The Ec 3, Ec 5 and Ec 6 isolates were resistant to 3rd generation agents, such as cefotaxime and ceftazidime. As for 4thG agents, the strains Ec 2, Ec 3, Ec 4, Ec 5, Ec 6, Ec 7 and Ec 9 grew around the cefepime disk. The *E. coli* antimicrobial resistance profile shows that the studied isolates have resistance mechanisms towards this antibiotic family effect, therefore suggesting the presence of β-lactamases-encoding genes. In order to investigate the mentioned resistance genes in these strains, a PCR sequence typing technique was performed. The results were the following, as shown in table 10:

- Six isolates harboured the *bla*$_{TEM}$ gene - Ec 1, Ec 2, Ec 4, Ec 7, Ec 8 and Ec 9
- Three harboured the *bla*$_{CTX-M-15}$ gene - Ec 1, Ec 5 and Ec 6
- Two harboured the *bla*$_{CTX-M-32}$ gene - Ec 2 and Ec 3
- Three harboured the *bla*$_{OXA-1}$ gene - Ec 3, Ec 5 and Ec 6.

Paterson & Bonomo (2005) mention that antimicrobial resistance profile demonstrated by bacteria depends on the harboured genes. If, for example, an isolate presented simultaneously CTX-M-type encoding genes and SHV-type encoding genes or CTX-M-type and AmpC-type encoding genes, different phenotypes would be expressed.
The *bla*$_{TEM}$ gene is associated to the production of β-lactamases. The enzymes produced by the variants TEM-1, TEM-2 and TEM-13 are considered Narrow Spectrum β-lactamases and grant resistance to ampicillin, carbenicillin, oxacillin and cephalotin. However, some TEM-variants grant an extended resistance to all β-lactams – such as those derivative from the NSBLs variants TEM-1 and TEM-2. In the isolates Ec 1, Ec 2, Ec 4, Ec 7, Ec 8 and Ec 9 the variant of the TEM could not be identified. Despite having different resistance profiles, all isolates demonstrated resistance to β-lactams. Further sequence typing would be helpful, in order to determine whether the harboured genes are responsible for the production of narrow

spectrum or extended spectrum β-lactamases. If the former option is true, an overproduction of these narrow spectrum enzymes could grant the resistance profile obtained in this experiment. An overproduction happens as a result of mutations occurring on the promoter region of the gene encoding TEM-1. Another plausible explanation for this event is that where the harboured genes are TEM-1 or TEM-2 derivatives, therefore ESBL-encoding genes (Kiiru, Kariuki, Goddeeris & Butaye, 2012).

The *bla*$_{\text{OXA-1}}$ gene encodes the OXA-1 enzymes, which have a low hydrolytic activity against the majority of β-lactams. It was identified in Ec 3, Ec 5 and Ec 6, isolates harbouring CTX-M-group 1 encoding genes as well, namely *bla*$_{\text{CTX-M-32}}$ and *bla*$_{\text{CTX-M-15}}$CTX-M-type enzymes are considered ESBLs, and therefore are effective inhibitors of a wider spectrum of β-lactams, in comparison to NSBLs. Penicillins, cephalosporins and aztreonam are ineffective against CTX-M-type enzymes. The TEM and SHV-type extended spectrum β-lactamases have been replaced in the last decade for the CTX-M enzymes, and are disseminated worldwide. Infections caused by multiresistant agents raise concern - not only higher costs are involved in treating infected patients but also the administration of last resort antimicrobial agents, such as carbapenems, is crucial. (Vimont *et al.*, 2012). The hydrolytic profile of early CTX-M-producers comprises cephalosporins such as cefotaxime and ceftriaxone. Later cephalosporins, as it is the case of ceftazidime, were not inactivated by these ESBLs. However, mutations in the amino acid sequence of the ESBLs encoding genes extended the spectrum of these enzymes. Some variants of the CTX-M family are capable of hydrolysing a broader spectrum of cephalosporins. CTX-M-32 is an enzyme derived from a single amino acid substitution. (Bush, 2010)

According to Baraniak *et al.* (2002) the *bla*$_{\text{CTX-M-15}}$ gene belongs to a specific group of CTX-M-type ESBLs that is associated to ceftazidime resistance. Jones-Dias *et al.* (2011) described the identification of the *bla*$_{\text{CTX-M-15}}$ in *E. coli* strains recovered from bottlenose dolphins' blowhole exudate in Portugal.

The production CTX-M-type extended-spectrum β-lactamases is in accordance with the resistance profiles of the isolates Ec 1, Ec 2, Ec 3, Ec 5 and Ec 6. The CTX-M-15 production by the strains Ec 5 and Ec 6 is in accordance with the resistance observed in the disk diffusion method to ceftazidime. Baraniak *et al.* (2002) and Vimont *et al.* (2012) are examples of authors describing urinary tract infections in human patients, associated with the isolation of pathogens carrying CTX-M-15 encoding genes.

The *E. coli* isolates recovered from dolphins harbour CTX-M-type ESBLs-encoding genes, raises uncertainty on the origin of these resistances. Further studies ought to be performed, in order to identify whether these resistances were transferred from humans to dolphins, or the other way around

According to Paterson & Bonomo (2005), ESBLs have hydrolytic activity against ceftazidime, cefotaxime, or aztreonam but are inhibited by clavulanic acid. The latter feature is not

inherent in AmpC β-lactamases. The isolate Ec 3 harboured both *bla*$_{CTX\text{-}M\text{-}32}$ and *bla*$_{DHA\text{-}1}$ genes. As seen in table 3, it was not inhibited by the amoxicillin and clavulanic acid association. This is an example of the resistance profile of an isolate producing simultaneously ESBLs and AmpC enzymes. ESBL and AmpC-producing strains have a wider resistance range to β-lactams agents, than a strain producing only one type of these enzymes. This association of resistance genes is responsible for high-level resistance to antibiotic treatment based on β-lactams. The resistance profile obtained through the disk diffusion method is consistent with the PCR findings. An infection caused by these multiresistant agents could be severe, and possibly life-threatening. (Paterson & Bonomo, 2005; Poole, 2004).

The presence of ESBLs in the *E. coli* strains explains the β-lactams resistances observed in the antimicrobial susceptibility test. The introduction of 3rd generation cephalosporins aimed to fight bacteria that were resistant to early cephalosporins, due to the fact that the presence of β-lactamases, in certain microorganisms, was increasing. Also, using these agents was seen as preventive measure on the ESBLs spreading into new hosts. However, the selective pressure exerted by the introduction of these broad-spectrum agents was responsible for the appearance of improved resistance genes, capable of producing extended spectrum enzymes (Paterson & Bonomo, 2005)

In conclusion, the obtained resistance profiles could be the result of selective pressure originated by the use of broad-spectrum antimicrobial agents in the past in these dolphins. It is uncertain whether the use of some of them has induced these mechanisms of resistance, but it is interesting to observe and compare the antimicrobial agents used in the dolphins of this study. Another explanation for the resistances found could be the introduction of β-lactamases by external vectors, such as staff or other animals of the park. Baraniak *et al.* (2002) also refers food supply as a potential reservoir of resistant bacteria and genetic determinants. Therefore fish could also be the origin of these resistance genes identified in the studied bacterial strains.

According to Szmolka & Nagy (2013) the presence of the ESBLs coding genes represent a public health risk. The carbapenems are potent β-lactams often used to treat infections caused by ESBL-producing bacteria. These agents are stable in the presence of common plasmid β-lactamases such as those derived from TEM, SHV-1, OXA and PSE enzymes, found in the Enterobacteriaceae family. However, carbapenems are only available for human use, reserved for hospitalized patients infected with multidrug-resistant bacteria.

In this study, the Ec 3 and Ec 5 isolates demonstrated to be clinically resistant to meropenem. The isolates Ec 2, Ec 5 and Ec 6 demonstrated to be intermediate resistant to meropenem and/or ertapenem. Also, epidemiological data suggests that all the nine isolates

are non-wild type towards meropenem, and the isolates Ec 2, Ec 3, Ec 4, Ec 5, Ec 6 and Ec 8 are non-wild type for ertapenem. The Ec 2 isolate demonstrated to be non-wild type to imipenem, as well. The presence of resistance genes to carbapenems was not tested, however, the results of antimicrobial susceptibility testing are suggestive of the production of carbapenemases. Therapeutic options against carbapenemases-producing bacteria, in case of infection, are limited (Poole, 2004).

Bacterial strains with resistance to β-lactams associated with the production of ESBL, usually possess resistance towards other families of antibiotics, such as aminoglycosides and fluoroquinolones. All the studied isolates were resistant to tetracylines and quinolones (nalidixic acid, ciprofloxacin, enrofloxacin and levofloxacin). According to the epidemiological breakpoints, these were considered non-wild type for the quinolones tested. Poole (2004) refers that most ESBL-producing bacteria are usually co-resistant to other antibiotic families, as a result of the ESBLs production in the Enterobacteriaceae family. Fluoroquinolones and aminoglycosides are usually the families to which these bacteria are resistant, other than β-lactams. This is the due to the fact that these antimicrobial resistance genes in the same mobile elements as the genes responsible for the ESBLs production.

Aminoglycosides

All of the studied isolates demonstrated resistance to the aminoglycoside antibiotics tested. The isolates Ec 3, Ec 5 and Ec 6 were considered resistant to gentamicin and tobramycin. The Ec 3 was resistant to amikacin, as well. The Ec 6 isolate was not inhibited by the disks containing amikacin and kanamycin. In order to investigate the presence of aminoglycoside resistance genes aaC(6') and aaC(3') in these resistant strains, the PCR technique was performed, identifying the *aac(6')-Ib* gene. The *aac(6')-Ib* gene codes for an N-acetyltransferase, that is capable of deflecting the aminoglycosides agents. In the group AAC(6') is comprised the *aac(6')-Ib* gene, which encodes more enzymes than any other AAC group. The AAC(6')-I enzymes modify amikacin and gentamicin while AAC(6')-II enzymes are active against gentamicin. (Shaw et al., 1993). The PCR findings are consistent with the resistance profiles obtained. According to the epidemiological breakpoints, the isolates Ec 3, Ec 5 and Ec 6 were considered NWT towards gentamicin, amikacin and tobramycin. The *aac(3')-Ib* gene was not harboured by any of the studied strains.

After analyzing the results from the current study it is accurate to say that the *E. coli* isolates are multidrug-resistant. Multidrug-resistant bacteria are able to inactivate at least one antimicrobial agent from more than three distinct families of antibiotics. The major concern of broad spectrum resistance profiles in bacteria is the therapeutic limitations it imposes. (Poole, 2004)

The confirmation of the presence of some resistance genes in several isolates, suggests that a continuous development or acquirement of new resistances, either after exposure to ineffective antimicrobial therapy and genetic transmission between bacteria or from contact with external vectors such as the staff or fomites. Paterson and Bonomo (2005) mention that human patients colonized or infected with ESBL producers do not demonstrate higher mortality rates, however their recovery is longer than those patients who did not get colonized or infected by non-ESBL-producing organisms.

2.1.2. *Morganella morganii*

The results from the *M. morganii* isolates are resumed in table 4, where both clinical and epidemiological resistance can be seen. The parameters used are presented in table 11, as well as the strains' classification, according to the inhibition zone diameters observed.

Table 4 - Resistance profile from the *Morganella morganii* strains

Isolate	*Clinical Resistances Profile*	*Epidemiological Resistance Profile*	*Classification according to the EUCAST parameters (in antibiotic families)*
Mm1	$Aml^{R}Amc^{R}Cxm^{R}Cl^{R}Kf^{R}Na^{R}S_3^{R}$ Fos^{R} <u>$Ipm^{I}Mem^{I}W^{I}$</u>	$KF^{NWT}Cl^{NWT}Fox^{NWT}$ $S3^{NWT}Na^{NWT}W^{NWT}Fos^{NWT}$	Non wild type 5 Clinical resistance 5
Mm2	$Aml^{R}Amc^{R}Cxm^{R}Cl^{R}Kf^{R}Fos^{R}$ <u>Mem^{I}</u>	$KF^{NWT}Cl^{NWT}Cxm^{NWT}Fox^{NWT}Fos^{NWT}$	Non wild type 3 Clinical resistance 3
Mm 3	$Aml^{R}Amc^{R}Ctx^{R}Cxm^{R}Cl^{R}Kf^{R}Fos^{R}$ <u>$Mem^{I}W^{I}$</u>	$KF^{NWT}Cl^{NWT}Ctx^{NWT}$ $Caz^{NWT}Fos^{NWT}$	Non wild type 2 Clinical resistance 3
Mm 4	$Aml^{R}Amc^{R}Cxm^{R}Cl^{R}Kf^{R}Fos^{R}$ <u>Mem^{I}</u>	$^{T}KF^{NWT}Cl^{NWT}Fox^{NWT}$ Fos^{NWT}	Non wild type 3 Clinical resistance 3

Abbreviations: Mm – *Morganella morganii*; Aml – Amoxicillin, Amc – Amoxicillin+clavulanic acid; Na – Nalidixic acid; Cl – Cephalexin; Kf – Cephalotin; Ctx – Cefotaxime; Fox – Cefoxitine; Caz – Ceftazidime; Cxm – Cefuroxime; S_3 - Sulfonamides compounds; Fos - Fosfomycin; According to EUCAST Clinical Breakpoints: R – clinically resistant; I – clinically intermediate. According to EUCAST Epidemiological cut-offs: NWT – Non Wild Type.

According to Barroso, Freitas-Vieira & Duarte (1999) *M. morganii* is a bacterial species intrinsically resistant to ampicillin, amoxicillin-clavulanic acid combination and 1st and 2nd generation cephalosporins. As for other antimicrobial agents, Mm 1, Mm 2 and Mm 4 were not inhibited by fosfomycin; and strain Mm 1 demonstrated resistance as well to nalidixic acid

(quinolone), sulfametoxazole and trimethoprim. Very few epidemiological cut-offs were available to analyse the inhibition zone diameters. However, the Mm 1 isolate was considered non-wild type to nalidixic acid, which is in accordance with the clinical resistance demonstrated; Mm 2 and Mm 4 are considered non-wild type to cefuroxime which is in accordance with the resistance profile demonstrated to this agent; Mm 3 demonstrated to be non-wild type to cefotaxime, cefoxitin, ceftazidime and cefuroxime, however this isolate was clinically susceptible to cefoxitin and ceftazidime. The resistance profile and epidemiological classification demonstrate that these strains are resistant to β-lactam antibiotics, information that ought to be considered before instituting an antimicrobial therapy based on such agents. The intermediate clinical resistance to meropenem and imipinem could not be appreciated in terms of mechanism of resistance. However, the administration of carbapenems in case of an infection caused by *M. morganii* should be avoided.

2.1.3. *Klebsiella oxytoca*

The clinical resistances profile of the *K. oxytoca* isolates are resumed in table 5. The parameters used are presented in table 12, as well as the strains' classification, according to the inhibition zone diameters observed.

Table 5- Resistance profile from the *Klebsiella oxytoca* strains

	Clinical Resistance profile	***Classification according to the EUCAST parameters (in antibiotic families)***
Ko1	$Aml^{R}Cl^{R}$	Clinical resistance 2
Ko 2	$Aml^{R}Tob^{R}Sxt^{R}W^{R}$	Clinical resistance 3

Abbreviations: Ko – *Klebsiella oxytoca* isolate; Aml – Amoxicillin; Cl – Cephalexin; Tob – Tobramycin; Sxt – Sulphametoxazole + trimethoprim; W – Trimethoprim. R – Resistant according to EUCAST's Clinical Breakpoints; According to EUCAST Clinical Breakpoints: R – Resistant.

Similarly to *M. morganii*, the *Klebsiella oxytoca* is intrinsically resistant to amoxicillin (Stock & Wiederman, 2001). This is consistent with the obtained resistance profile. The Ko 1 isolate was not inhibited by amoxicillin, as expected, neither by cephalexin. The isolate Ko 2 demonstrated a broader spectrum of resistance, not being inhibited by tobramycin (aminoglycoside), by trimethoprim and as well to its association with sulfamethoxazole.

The current study did not comprise the identification of resistance genes in the isolates of *K. oxytoca* and *M. morganii*, however ESBLs have been commonly identified in these species,

in the past, as well (Paterson & Bonomo, 2005; Poole, 2004). Thus, it can be said that the concerns expressed for the ESBLs-producing *E. coli* are as well emergent in these other bacterial species, from the Enterobacteareacea family.

2.2. Enterococcus faecalis

The antimicrobial resistance was studied in the *E. faecalis* through antimicrobial susceptibility testing, using the disk diffusion method. Thirteen antimicrobial agents were tested, and the obtained results can be seen in table 6. The parameters used are presented in table 13, as well as the strains' classification, according to the inhibition zone diameters observed.

Table 6 - Resistance Profile from the Enterococcus faecalis strains

	Clinical resistances profile	***Epidemiological Resistance Profile***	***Classification according to the EUCAST parameters (in antibiotic families)***
Ef1	$Te^{R}Str^{R}Lev^{R}Da^{R}E^{R}$ $Cn^{I}C^{I}$	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5
Ef2	$Te^{R}Str^{R}Lev^{R}Da^{R}E^{R}$ $Cn^{I}C^{I}$	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5
Ef3	$Te^{R}Str^{R}Lev^{R}Da^{R}E^{R}$ Cn^{I}	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5
Ef4	$Te^{R}Cn^{R}Str^{R}Lev^{R}Da^{R}E^{R}$	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5
Ef5	$Te^{R}Str^{R}Lev^{R}Da^{R}E^{R}$ $Cn^{I}C^{I}$	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5
Ef6	$Te^{R}Str^{R}Lev^{R}Da^{R}E^{R}$ Cn^{I}	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5
Ef7	$Cn^{R}Str^{R}Da^{R}$ $E^{I}C^{I}$	$\underline{Da^{NWT}Str^{NWT}}$	Non wild type 2 Clinical resistance 2
Ef8	$Te^{R}Cn^{R}Str^{R}Lev^{R}Da^{R}E^{R}$ C^{I}	$\underline{Te^{NWT}Lev^{NWT}Da^{NWT}E^{NWT}Str^{NWT}}$	Non wild type 5 Clinical resistance 5

Abbreviations: Ef– *Enterococcus faecalis* isolate; Te – Tetracycline; Cn – Gentamycin; Lev – Levofloxacin; Da – Clindamycin; E – Erythromycin; Lzd – Linezolid. Str – Streptomycin R – Resistant according to EUCAST's Clinical Breakpoints. NWT – non wild type

The *Enterococcus faecalis* is a bacterial species intrinsically resistant to cephalosporins and several synthetic β-lactams, and to lincosamides as well (Quinn *et al.*, 2002). The resistance profile obtained through the disk diffusion method was similar in all the recovered isolates. The strains Ef 1, Ef 2, Ef 3, Ef 5 and Ef 6 were not inhibited by the following antimicrobial families: tetracyclines, lincosamides (clindamycin), fluoroquinolones (levofloxacin) and macrolides (erithromycin). All strains demonstrated to be resistant to the clindamycin disk. The resistance to lincosamides is not associated to the acquisition of resistance mechanisms, in that it is a natural resistance in this bacterial species. As for the resistance to aminoglycoside agents, the gentamycin disk demonstrated different inhibition zone diameters in all isolates. The isolates Ef 4, Ef 7 and Ef 8 were classified as resistant to this agent. The other isolates were considered to have intermediate resistance to it. However, another aminoglycoside agent was tested against the *E. faecalis* isolates – streptomycin. In table 6 these three strains are classified as non-wild type and clinically resistant to streptomycin.

One of the emergent concerns in enterococci is the acquisition of vancomycin-resistance mechanisms. This is particularly concerning when occurring in enterococci strains resistant to other antimicrobial families, such as aminoglycosides (high level aminoglycoside resistance - HLAR), β-lactams and glycopeptides. However, it seems that this is a resistance usually associated to the *E. faecium* species. (Marothi, Agnihotri & Dubey, 2005).

In the disk diffusion method these strains showed resistance to the aminoglycosides tested, with small inhibition zone diameters (10-12 mm) around the disk of tobramycin. However both clinical breakpoints and epidemiological cut-offs for tobramycin are not available in the EUCAST web site neither in the CLSI document. Therefore, this resistance could not be confirmed. Other antimicrobial agents from this family should be tested in order to name these strains aminoglycoside-resistant, however the results regarding gentamycin and streptomycin are highly indicative of this. As for the vancomycin and teicoplanin results (glycopeptides family), all strains demonstrated full susceptibility, thus these enterococci were not vancomycin-resistant.

2.3. *Staphylococcus spp*.

Methicillin resistance test results

Three of the six staphylococci strains tested demonstrated a significant growth on the selective media, as seen in figure 10. This technique is an early step in antimicrobial susceptibility testing, allowing a triage and identification of methicillin-resistant staphylococci. These strains are capable of inactivating all β-lactams antibiotics and this selective media is a first indicator of the *mec*A or *mec*C genes presence (Neu, 1992). Significant growth occurred on the Brilliance^{TM} MRSA 2 agar (Oxoid®, Remel®, United Kingdom). Two blue colonies (*S. aureus*) and one white colony (*S. hominis*) were observed, after the incubation period.

Figure 10 - Methicilin-Resistant *Staphylococcus* spp. in Brilliance™ MRSA 2 agar (Oxoid®, Remel®, United Kingdom). Font: Machado, 2013)

Although this is a reliable test, the disk diffusion method and the PCR amplification for the *mec*A detection are essential to confirm the resistance profile of these strains as well as to identify the presence of resistant genetic elements.

In table 7 are presented the susceptibility testing results, as well as the PCR findings. The parameters used are presented in table 14, as well as the strains' classification, according to the inhibition zone diameters observed.

Table 7 - Compilation of the results from the staphylococci isolates

Isolate	**Clinical Resistance Profile**	**Epidemiological Resistance Profile**	***Classification according to the EUCAST parameters (in antibiotic families)***	**mecA**
Sa 1	$P^RAmp^ROx^RE^R$	E^{NWT}	Clinical resistance 2	*mec*A (+)
Sa 2	$P^RAmp^ROx^RE^R$	E^{NWT}	Clinical resistance 2	(-)
Sd	Fully susceptible			
Ss				
Shom	$P^ROx^RE^RFD^R$	NA	Clinical resistance 3	*mecA* (+)
Shaem	P^RAk^R	NA	Clinical resistance 2	

Sa – *Staphylococcus aureus*; Sd – *S. delphini*; Ss – *S. simulans*; Shom – *S. hominis*; Shaem – *S. haemolyticus.* P- Penicillin, Amp – Ampicillin, E – Erythromycin; Ox – Oxacillin; FD – Fusidic acid; AK – amikacyn; NA – data not available.R – Resistant according to EUCAST Clinical Breakpoints; NWT – Non Wild Type according to EUCAST Epidemiological cut-offs. *mecA*(+) positive, (-) negative.

The isolates of *S. simulans* and *S. delphini* were fully susceptible to all the 19 antimicrobial agents tested. The *S. hominis* isolate demonstrated a wider range of resistance not being inhibited by penicillins (penicillin, oxacillin), quinolones (fusidic acid) and macrolides (erythromycin). The epidemiological cut offs could not be applied to the results obtained in these strains, because no data is available at the EUCAST web site. Both the *S. aureus* isolates are resistant to penicillins (penicillin, ampicillin and oxacillin) and macrolides (erythromycin) and considered non-wild type to erythromycin by EUCAST ECOFFs. The *mec*A gene was identified in two strains, S. *aureus 1 and S. hominis*, through PCR amplification. Although it was expected to identify the *mec*A or *mec*C gene in both the *S. aureus* strains, as these strains grew on the Brilliance^{TM}MRSA 2 agar (methicillin resistant), the Sa 2 isolate did not harbour this gene.

Paterson and Bonomo (2005) mention the case of MRSAs in which the methicillin resistance occurs without the presence of the *mec*A gene, and this could be due to alterations/overproduction of other PBP's on the resistant bacteria.

Staphylococcus aureus is not only an important pathogen threatening domestic animals but also wildlife. Van Elk *et al.* (2012) explains that these strains have become host species specific, as a result of the co-evolution with the hosts. According to O'Mahony *et al.* (2005) methicillin-resistant *S. aureus* infections have been described in animals for the first time in 1972, and since then in several other domestic species such as dogs, cats, cattle, sheep, chickens, rabbits and horses. Faires, Gehring, Mergl & Weese (2009) are the authors of a study carried out at a marine park in North America, in which the isolation of an MRSA from three bottlenose dolphins under human care is described. However, in Europe MRSA strains in cetaceans had not been described until now.
The presence of the *mec*A gene confers resistance essentially to penicillins, cephalosporins, carbapenems and monobactams, however MRSA may harbour as well additional antibiotic resistance genes which contributes to the multidrug resistance described for MRSA. (Neu, 1992)

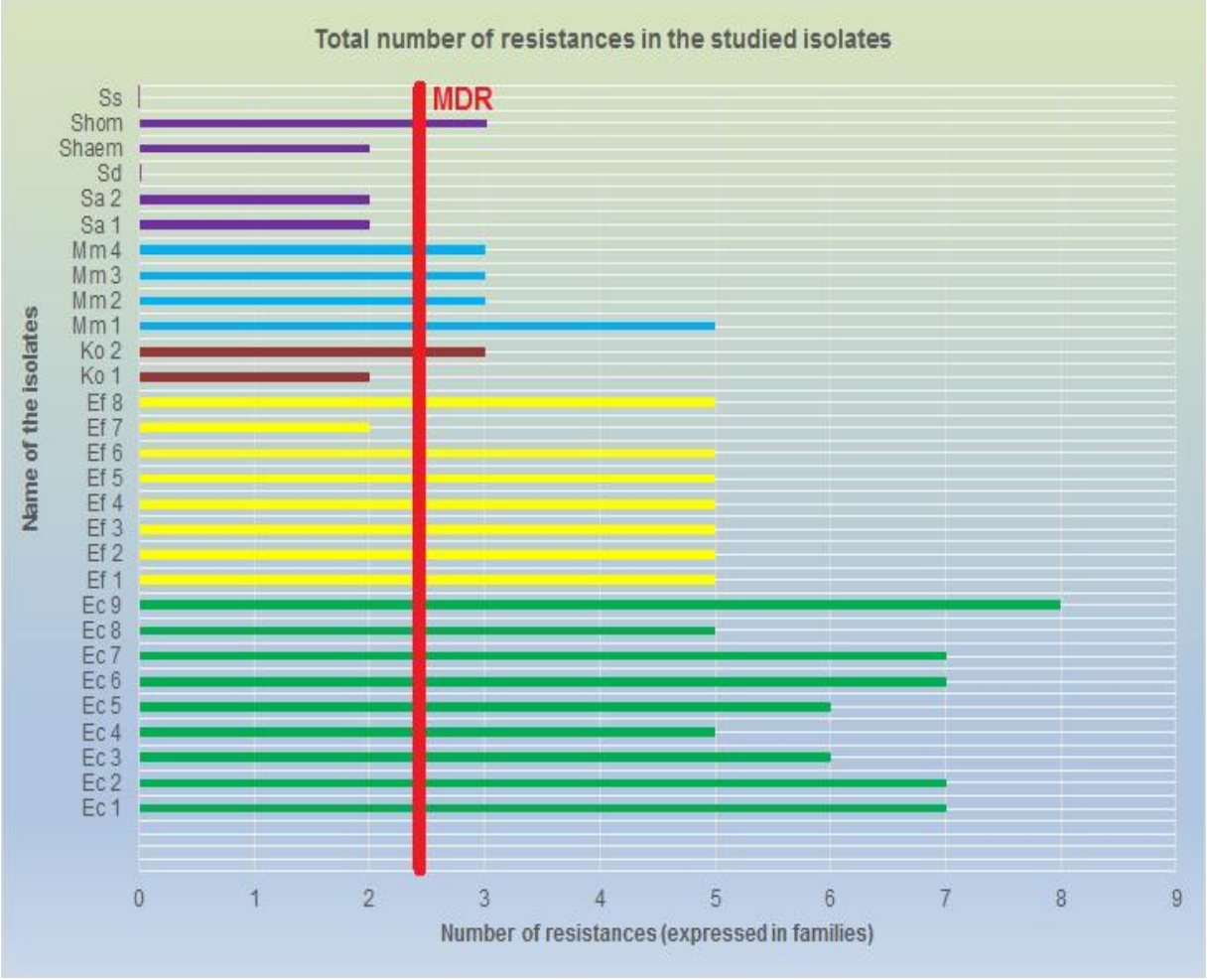
MRSA can also demonstrate resistance mechanisms to other antibiotics such as erythromycin, fusidic acid, tetracycline, minocycline, streptomycin, spectinomycin and sulfonamides (Fuda, Suvorov, Vakulenko & Mobashery, 2004). This information is in agreement with this study's results, in that the MRS isolates harbouring the *mec*A gene were also resistant to erythromycin and in the case of *S. hominis* also to fusidic acid and tetracycline. However, only the *S. hominis* isolate can be considered multidrug resistant, while the Sa 1 isolate only demonstrated to be resistant to two antibiotic families. Neu (1992) refers that some coagulase-negative staphylococci are also β-lactamase producers and

many possess the PBP2a. The presence of the mecA gene on the *S. hominis* isolate, reinforces the idea that CNS species can act as reservoirs of the SCCmec and can be transferred horizontally to other staphylococci, contributing to the spread of the *mec*A gene.

Multidrug resistance

In chart 3 is demonstrated the total number of resistances found in all the isolates studied. This chart is organized through the number of antibiotic families to which each strain was clinically resistant. The numbers in the "x" axis are correspondent to the number of antibiotic families and in the "y" axis are the names of the different isolates studied. The label MDR allows the differentiation between the group of multidrug resistant strains and those fully susceptible or resistant to one or two families of antibiotics.

Chart 3 - Total number of resistances from the studied isolates



Ec1-Ec9 – *E. coli*; Ef1-Ef8 – *E. faecalis*; Mm1-Mm4 – *M. morganii*; Shom – *S. hominis*; Ko1-2 – *K. oxytoca;* Shaem – *S. haemolyticus*; Sa1-2 – *S. aureus;* Ss – *S. simulans;* Sd – *S. delphini.* MDR label – Multidrug resistance

From the 29 isolates studied, 7% were fully susceptible (n=2) which means these are inhibited by all the tested antimicrobial agents (*S. simulans* and *S. delphini*). Seventeen percent (n=5) of the total isolates represented by staphylococci and Enterobacteriaceae were resistant to one or two families of antibiotics ($1 \leq x \leq 2$) and 76% of the total isolates (n=22) were multidrug resistant strains which means these were not inhibited by one or more agents from three or more antibiotic families. This majority is represented mostly by the *Enterococcus faecalis* and *E. coli* species.

In the wild, multidrug resistance in bacteria recovered from top-level marine predators was described by Blackburn (2003). The author identified MDR strains in animals inhabiting Florida, Belize, Florida Keys and Louisiana waters, concluding that a continuous monitoring of sentinel wildlife is essential to analyse the potential risks concerning public health. Although the current study approaches antimicrobial resistance in dolphins under human care, it is important to highlight that bacterial strains recovered from free-range dolphins seem to possess the same acquired resistances to antimicrobial agents (Greig, Bemiss, Lyon, Bossart & Fair, 2007).

The MRSA isolation raises concern; however, further studies should be performed in order to obtain additional information. In a future study, recent samples should be used in order to revaluate this situation and the new sampling methods ought to be studied before the collection of the *sputum*. These results demonstrate the need of performing new microbiological screenings and antimicrobial susceptibility tests in all animals, surrounding environment and humans in contact. MRSA in the studied dolphins and/or in their environment should be monitored in order to confirm its presence. In that case it would be essential to adopt a series of preventive measures in order to avoid its transmission and consider changes in antibiotic therapy instituted thenceforward in all animals.

According to Faires *et al.* (2009), the transient carriage on the hands of the staff in direct contact with infected animals is an important means of transfer and could be eliminated by hands disinfection with biocides such as chlorhexidine or alcohol based antiseptics. Thus, it is essential to sensitize the park staff to thoroughly adopt adequate hygienic habits.

Faires *et al.* (2009) refers that the decolonization of MRSA is common. The use of antimicrobial agents to eradicate these strains is discouraged (Faires *et al.*, 2009).

These animals are closely monitored and when any subtle clinical sign of disease is detected an immediate health status screening is performed in order to prevent its progression. The veterinary services major concern is to guarantee animal welfare and this is possible due to a complex monthly medical programme that includes blood screening, respiratory cytology among other exams.

Unfortunately, due to these species' higher incidence of respiratory diseases, it is essential to understand which antimicrobial agents are more adequate and therefore electable for therapy in infectious processes. The results obtained in this study raise concern in that a significant quantity of antimicrobial resistances was found.

The results from the current study demonstrated that the isolated bacterial species have a generalized resistance profile, with the exception of *Klebsiella oxytoca*, *M. morganii* and some of the staphylococci isolates. Concerning the studied *E. coli* strains, these weren't inhibited by aminoglycoside agents, and neither was the majority of the isolates by β-lactams, such as the association amoxicillin-clavulanic acid and cephalosporins. A generalized resistance profile to tetracyclines, quinolones and carbapenems was verified in the majority of the isolates from this bacterial species. Most of the *Enterococcus faecalis* isolates were resistant to aminoglycosides, lincosamides, tetracyclines, fluoroquinolones, macrolides and oxazolidinones. The available antimicrobial agents in the Zoomarine's veterinary hospital are amoxicillin-clavulanic acid, cephalosporins, such as cephalexin, cefuroxime and ceftriaxone, quinolones, such as enrofloxacin, doxicicline, ciprofloxacin, aminoglycosides, such as clindamycin, and sulphonamide-trimethoprim. Most of these agents were used previously against bacterial infections, in the sampled bottlenose dolphins. The resistances observed in the disk diffusion method are related to the antibiotic use in the animals, suggesting that acquisition of resistance, by bacteria, could have been promoted by the use of these agents.

According to Goldstein, Schaefer, McCulloch, Fair, Bossart and Reif (2012), interpreting this type of findings requires caution, in that examinations performed in a single point in time, are not as accurate as serial investigations. Ideally, further information on antimicrobial resistance in these animals should be gathered, meaning that a full screening of all animals, staff and environment should be carried out.

V. Conclusion

The commensal microbiota identified in the current study, was in accordance with the results described by previous authors, highlighting that the bacterial species isolated are usually present in healthy animals. The fact that these microorganisms are often isolated in cases of pneumonia, is suggestive of their pathogenic role, especially in immune depressed individuals. Antimicrobials resistance is an emergent topic in veterinary medicine. Several antimicrobial agents were tested in all the isolates recovered from the URT of these bottlenose dolphins. The results demonstrated that most of the strains were resistant to, at least, one antimicrobial agent. A percentage of 76 isolates were considered MDR, 17% resistant (resistance to one/two antibiotic families) and only 7% of the isolates were susceptible to all the agents tested. This is a major problem in that any infection caused by these agents would be resistant to therapy. Significant findings were the isolation of an MRSA from one animal and the identification of the $bla_{\text{CTX-M-15}}$ in some of the *E. coli* isolates.

This raises concern in that it demonstrates how widespread these resistances are. A new screening of all animals, their environment and park staff is recommended in order to evaluate the present situation. However, if the results from the current study are consistent with future findings, preventive measures should be adopted in order to eradicate MRSA strains.

The major concern on the results obtained is the risk of infection by these strains. Although these were present in healthy dolphins, and not causing disease at the time of the blowhole exudate collection, alterations of the microbiota or in the host health-status may lead to multidrug resistant respiratory infections. As previously referred, antibacterial research activity has decreased in the past years alongside with technological advances, concerning the development of new activity mechanisms antimicrobial agents, which represents another limitation to eliminate multiresistant bacteria. Interventional measures ought to be adopted in this park in order to eliminate multidrug resistant bacteria and preventive measures to prevent the appearance of new resistances.

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VII. Attachments

Table 8– List of the Primers required for the different Polymerase Chain Reaction protocols. Font: UL/FMV LRAB data base

Primers	Nucleotide Sequence (5'-3')	PCR Product Size	Reference
mecA-1	GGGATCATAGCGTCATTATTC	527	**EURL**
mecA-2	AACGATTGTGACACGATAGCC		
nuc-1	TCAGCAAATGCATCACAAACAG	255	
nuc-2	CGTAAATGCACTTGCTTCAGG		
16S-1	GTGCCAGCAGCCGCGGTAA	886	
16S-2	AGACCCGGGAACGTATTCAC		
mecLGA251f	GCTCCTAATGCTAATGCA	340	**Cuny et al 2011 PLoS 6(9): e24360 bvvv**
mecLGA251f	TAAGCAATAATGACTACC		
dea-F	TGAAGGCATATTGTAGAACAA	661	**Sasaki et al 2010 JCM, 48(3): 765-769**
dea-R	CGRTACTTTTCGTTAGGTCG		
deb-F	GGAAGRTTCGTTTTTCCTAGA	1135	
deb-R4	TATGCGATTCAAGAACTG		
ctx-m-1-seqf	GGTTAAAAAATCACTGCGTC	863	**Sundsfjord et al 2004 APMIS 112**
ctx-m-1-seqr	TTGGTGACGATTTTAGCCGC		
MOXMF	GCTGCTCAAGGAGCACAGGAT	520	
MOXMR	CACATTGACATAGGTGTGGTGC		
CITMF	TGGCCAGAACTGACAGGCAAA	462	
CITMR	TTTCTCCTGAACGTGGCTGGC		
DHAMF	AACTTTCACAGGTGTGCTGGGT	405	
DHAMR	CCGTACGCATACTGGCTTTGC		
ACCMF	AACAGCCTCAGCAGCCGGTTA	346	
ACCMR	TTCGCCGCAATCATCCCTAGC		
EBCMF	TCGGTAAAGCCGATGTTGCGG	302	
EBCMR	CTTCCACTGCGGCTGCCAGTT		
FOXMF	AACATGGGGTATCAGGGAGATG	190	
FOXMR	CAAAGCGCGTAACCGGATTGG		
AAC6Ibf3	TTGCGATGCTCTATGAGTGG	482	
AAC6Ibr3	CTCGAATGCCTGGCGTGTTT		
AacC4f	CTTCAGGATGGCAAGTTGGT	286	**Sáenz Y et al AAC 2004 48 3996 (C. Torres)**
AacC4r	TCATCTCGTTCTCCGCTCAT		

Primers	Nucleotide Sequence (5'-3')	PCR Product Size	Reference
P1	TACGATACGGGAGGGCTTAC	716	**Pomba *et al.*, 2006**
P2	TTCCTGTTTTTGCTCACCCA		
shvf1	TCAGCGAAAAACACCTTG	471	
shvr	TCCCGCAGATAAATCACCA		
AmpCfor634	CCCCGCTTATAGAGCAACAA	634	
AmpCrev634	TCAATGGTCGACTTCACACC		
Oxa1f	TATCTACAGCAGCGCCAGTG	199	
Oxa1r	CGCATCAAATGCCATAAGTG		

Table 9 - Disk selection for the disk diffusion method, according to the bacterial species studied

Antimicrobial agents	Disk content (µg)	*Enterobactereacea*	*Staphylococcus spp.*	*Enterococcus faecalis*
Nalidixic acid	30	X		
Fusidic acid	10		X	
Amikacin	30	X	X	
Amoxicillin	25	X		
Amoxicillin&Clavulanic Acid	20 + 10	X	X	
Ampicillin	10		X	X
Cefalexin	30	X		
Cefalotin	30	X	X	
Cefepime	30	X		
Cefotaxime	30	X	X	
Cefoxitine	30	X		
Ceftazidime	30	X		
Cefuroxime	30	X		
Ciprofloxacin	5	X		
Clindamycin	2		X	X
Chloramphenicol	30	X		X
Enrofloxacin	5	X	X	
Erythromycin	15		X	X
Ertapenem	10	X		
Streptomycin	10			X
Florphenicol	30	X	X	
Fosfomycin	200	X	X	
Gentamicin	120	X	X	X
Imipenem	10	X		
Kanamycin	30	X		
Levofloxacin	5	X		X
Linezolide	30			X
Meropenem	10	X		
Moxifloxacin	5			X
Nitrofurantoin	300			X
Oxacillin	1		X	
Penicillin	10		X	
Compound Sulfonamides	300	X		
Teicoplanin	30			X
Tetracycline	30	X	X	X
Tobramycin	10	X		
Trimethoprim + Sulfametoxazole	1.25 + 23.75	X	X	X
Trimethoprim	5	X		
Vancomycin	30			X

Table 10 - Antimicrobial susceptibility test Results – inhibition Zone Diameter observed in the *E. coli* strains (In milimeters)

Abbreviations: NA – Nalidixic Acid; AK – Amikacin, AM – Amoxicillin, AMC – Amoxicillin + Clavulanic Acid; CL – Cephalexin; KF – Cephalotin; FEP – Cefepime; CTX – Cefotaxime; FOX – Cefoxitin, CAZ – Ceftazidime; CXM – Cefuroxime; CIP – ciprofloxacin; C – Choramphenicol; ENR – enrofloxacin; ETP – Ertapenem; FFC – Florphenicol; FOS - Fosfomycin, CN – Gentamycin; IPM – Imipenem; K – Kanamycin; LEV – Levofloxacin; MEM – Meropenem: S_3 - Sulfonamides Compounds; TE – Tetracycline; TOB- Tobramycin; SXT – Sulphonamide+Trimethoprim; W – Trimethoprim. IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI M31-A3

Strain	NA			AK			AML			AMC			CL			KF			FEP			CTX		
	IZD	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB [b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB [b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**
Ec 1	0	R	NWT	20	S	WT	0	R	NWT	22	S	WT	0	R	NWT	18	S	NA	26	S	NWT	0	R	NWT
Ec 2	0	R	NWT	18	S	WT	0	R	NWT	26	S	WT	0	R	NWT	14	R	NA	20	R	NWT	29	S	WT
Ec 3	0	R	NWT	16	S	WT	0	R	NWT	0	R	NWT	0	R	NWT	0	R	NWT	10	R	NWT	0	R	NWT
Ec 4	0	R	NWT	20	S	WT	0	R	NWT	18	S	WT	0	R	NWT	18	I	NA	24	S	NWT	34	S	WT
Ec 5	0	R	NWT	18	S	WT	0	R	NWT	12	R	NWT	0	R	NWT	0	R	NWT	12	R	NWT	0	R	NWT
Ec 6	0	R	NWT	14	I	WT	0	R	NWT	8	R	NWT	0	R	NWT	0	R	NWT	8	R	NWT	0	R	NWT
Ec 7	0	R	NWT	22	S	WT	0	R	NWT	16	R	NWT	8	R	NWT	16	I	NA	24	S	NWT	32	S	WT
Ec 8	0	R	NWT	18	S	WT	0	R	NWT	18	S	WT	0	R	NWT	14	R	NA	30	S	WT	30	S	WT
Ec 9	0	R	NWT	22	S	WT	0	R	NWT	16	R	NWT	0	R	NWT	14	R	NA	24	S	NWT	32	S	WT
CB/ ECOFF		**S≥19 R<13**	**≥19**		**S≥16 R<8**	**≥8**		**S≥21 R<13**	**ND**		**S≥17 R<17**	**≥17**		**S≥14 R<14**	**≥14**		**S≥18 R<14**	**ND**		**S≥24 R<21**	**≥28**		**S≥20 R<17**	**≥23**

Strain	FOX			CAZ			CXM			CIP			C			ENR			ETP			FFC		
	IZD	**CB[b]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB**	**ECOFF**
Ec 1	24	S	WT	28	S	WT	18	S	WT	0	R	NWT	20	S	WT	0	R	NWT	30	S	WT	20	NA	NA
Ec 2	22	S	WT	28	S	WT	16	R	NWT	0	R	NWT	20	S	WT	0	R	NWT	24	I	NWT	22	NA	NA
Ec 3	22	S	WT	10	R	NWT	0	R	NWT	0	R	NWT	20	S	WT	0	R	NWT	24	I	NWT	18	NA	NA
Ec 4	26	S	WT	30	S	WT	14	R	NWT	0	R	NWT	16	R	NWT	0	R	NWT	28	S	NWT	16	NA	NA
Ec 5	26	S	WT	10	R	NWT	0	R	NWT	0	R	NWT	26	S	WT	0	R	NWT	24	I	NWT	22	NA	NA
Ec 6	24	S	WT	0	R	NWT	0	R	NWT	0	R	NWT	20	S	WT	0	R	NWT	22	I	NWT	18	NA	NA
Ec 7	24	S	WT	30	S	WT	20	S	WT	0	R	NWT	22	S	WT	0	R	NWT	30	S	WT	22	NA	NA
Ec 8	22	S	WT	28	S	WT	16	R	NWT	0	R	NWT	20	S	WT	0	R	NWT	28	S	NWT	18	NA	NA
Ec 9	24	S	WT	28	S	WT	14	R	NWT	0	R	NWT	16	R	NWT	0	R	NWT	30	S	WT	18	NA	NA
CB/ ECOFF		**S≥18 R<14**	**≥19**		**S≥22 R<19**	**≥22**		**S≥18 R<18**	**≥18**		**S≥22 R<19**	**≥25**		**S≥17 R<17**	**≥17**		**S≥23 R<16**	**NA**		**S≥25 R<22**	**≥29**		**NA**	**NA**

Abbreviations: NA – Nalidixic Acid; AK – Amikacin, AML – Amoxicillin, AMC – Amoxicillin+Clavulanic Acid; CL – Cephalexin; KF – Cephalotin; FEP – Cefepime; CTX – Cefotaxime; FOX – Cefoxitin, CAZ – Ceftazidime; CXM – Cefuroxime; CIP – Ciprofloxacin; C – Choramphenicol; ENR – Enrofloxacin; ETP – Ertapenem; FFC – Florphenicol; FOS - Fosfomycin, CN – Gentamycin; IPM – Imipenem; K – Kanamycin; LEV – Levofloxacin; MEM – Meropenem: S3 - Sulfonamides Compounds; TE – Tetracycline; TOB- Tobramycin; SXT – Sulfonamide+Trimethoprim; W – Trimethoprim.

Strain	FOS			CN			IPM			K			LEV			MEM			S3			TE		
Susceptibility evaluation	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**
Ec 1	24	S	NA	0	R	NWT	30	S	WT	16	I	NA	0	R	NWT	18	I	NWT	0	R	NWT	0	R	NWT
Ec 2	16	S	NA	0	R	NWT	23	S	NWT	10	R	NA	0	R	NWT	20	I	NWT	14	I	NA	0	R	NWT
Ec 3	12	I	NA	0	R	NWT	24	S	WT	6	R	NA	8	R	NWT	14	R	NWT	26	S	NA	0	R	NWT
Ec 4	22	S	NA	20	S	WT	30	S	WT	10	R	NA	0	R	NWT	16	I	NWT	14	I	NA	14	I	NA
Ec 5	10	R	NA	0	R	NWT	28	S	WT	8	R	NA	0	R	NWT	12	R	NWT	24	S	NA	18	I	NA
Ec 6	10	R	NA	0	R	NWT	28	S	WT	0	R	NWT	0	R	NWT	24	S	NWT	0	R	NWT	0	R	NWT
Ec 7	24	S	NA	0	R	NWT	28	S	WT	14	I	NA	0	R	NWT	20	I	NWT	0	R	NWT	0	R	NWT
Ec 8	16	S	NA	18	S	WT	30	S	WT	16	I	NA	0	R	NWT	18	I	NWT	20	S	NA	14	I	NA
Ec 9	16	S	NA	0	R	NWT	30	S	WT	10	R	NA	0	R	NWT	16	I	NWT	0	R	NWT	0	R	NWT
CB/ ECOFF		**S≥16 R<12**	**ND**		**S≥17 R<14**	**≥16**		**S≥22 R<16**	**≥24**		**S≥18 R<13**	**ND**		**S≥22 R<19**	**≥25**		**S≥22 R<16**	**≥25**		**S≥17 R<12**	**ND**		**S≥19 R<13**	

IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Strain	TOB			SXT			W		
Susceptibility evaluation	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**
Ec 1	10	R	NWT	0	R	NWT	0	R	NWT
Ec 2	9	R	NWT	28	S	WT	7	R	NWT
Ec 3	0	R	NWT	26	S	WT	16	I	NWT
Ec 4	16	I	WT	26	S	WT	18	S	NWT
Ec 5	0	R	NWT	26	S	WT	20	S	WT
Ec 6	0	R	NWT	0	R	NWT	0	R	NWT
Ec 7	8	R	NWT	0	R	NWT	0	R	NWT
Ec 8	9	R	NWT	13	I	NWT	7	R	NWT
Ec 9	8	R	NWT	0	R	NWT	0	R	NWT
CB/ ECOFF		**S≥17 R<14**	**≥16**		**S≥16 R<13**	**≥16**		**S≥18 R<15**	**≥20**

Table 11 -Antimicrobial susceptibility test Results – inhibition Zone Diameter observed in the *M. morganii* strains (In milimeters)

Abbreviations: NA – Nalidixic Acid; AK – Amikacin, AML – Amoxicillin, AMC – Amoxicillin+Clavulanic Acid; CL – Cephalexin; KF – Cephalotin; FEP – Cefepime; CTX – Cefotaxime; FOX – Cefoxitin, CAZ – Ceftazidime; CXM – Cefuroxime; CIP – Ciprofloxacin; C – Choramphenicol; ENR – Enrofloxacin; ETP – Ertapenem; FFC – Florphenicol; FOS - Fosfomycin, CN – Gentamycin; IPM – Imipenem; K – Kanamycin; LEV – Levofloxacin; MEM – Meropenem: S3 - Sulfonamides Compounds; TE – Tetracycline; TOB- Tobramycin; SXT – Sulfonamide+Trimethoprim; W – Trimethoprim.
IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data available

Strain	**NA**			**AK**			**AML**			**AMC**			**CL**			**KF**			**FEP**			**CTX**		
	IZD	**CB[b]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF[a]**
Mm 1	12	R	NWT	22	S	NA	8	R	NWT	0	R	NWT	0	R	NWT	0	R	NWT	30	S	NA	30	S	WT
Mm 2	26	S	WT	22	S	NA	0	R	NWT	0	R	NWT	0	R	NWT	0	R	NWT	32	S	NA	28	S	WT
Mm 3	26	S	WT	22	S	NA	0	R	NWT	0	R	NWT	0	R	NWT	0	R	NWT	32	S	NA	26	S	WT
Mm 4	24	S	WT	20	S	NA	0	R	NWT	0	R	NWT	0	R	NWT	0	R	NWT	32	S	NA	26	S	WT
CB/ ECOFF		**S≥19 R<13**	**≥19**		**S≥16 R<13**	**ND**		**S≥21 R<13**	**≥17**		**S≥17 R<17**	**ND**		**S≥14 R<14**	**ND**		**S≥18 R<14**	**ND**		**S≥24 R<21**	**ND**		**S≥20 R<17**	**≥23**

Strain	FOX			CAZ			CXM			CIP			C			ENR			ETP			FFC		
	IZD	**CB[b]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB**	**ECOFF**
Mm 1	18	S	NWT	28	S	NA	12	R	NA	28	S	NA	28	S	NA	26	S	NA	26	S	NA	22	NA	NA
Mm 2	18	S	NWT	30	S	NA	12	R	NA	34	S	NA	22	S	NA	26	S	NA	28	S	NA	26	NA	NA
Mm 3	20	S	WT	30	S	NA	10	R	NA	26	S	NA	24	S	NA	26	S	NA	26	S	NA	26	NA	NA
Mm 4	18	S	NWT	31	S	NA	12	R	NA	32	S	NA	22	S	NA	33	S	NA	26	S	NA	26	NA	NA
CB/ ECOFF		**S≥18 R<14**	**≥19**		**S≥22 R<19**	**ND**		**S≥18 R<18**	**ND**		**S≥22 R<19**	**ND**		**S≥17 R<17**	**ND**		**S≥23 R≤16**	**ND**		**S≥25 R<22**	**ND**		**ND**	**ND**

Strain	FOS			CN			IPM			K			LEV			MEM			S3			TE		
Susceptibility evaluation	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**
Mm 1	8	R	NA	22	S	NA	20	I	NA	22	S	NA	26	S	NA	18	I	NA	0	R	NWT	20	S	NA
Mm 2	10	R	NA	22	S	NA	22	S	NA	22	S	NA	26	S	NA	20	I	NA	18	S	NA	22	S	NA
Mm 3	8	R	NA	22	S	NA	22	S	NA	22	S	NA	28	S	NA	20	I	NA	20	S	NA	22	S	NA
Mm 4	0	R	NWT	22	S	NA	23	S	NA	22	S	NA	30	S	NA	16	I	NA	26	S	NA	23	S	NA
CB/ ECOFF		**S≥16 R<13**	**ND**		**S≥17 R<14**	**ND**		**S≥22 R<16**	**ND**		**S≥18 R<13**	**ND**		**S≥22 R<19**	**ND**		**S≥22 R<16**	**ND**		**S≥17 R<12**	**ND**		**S≥19 R<13**	**ND**

Abbreviations: NA – Nalidixic Acid; AK – Amikacin, AML – Amoxicillin, AMC – Amoxicillin+Clavulanic Acid; CL – Cephalexin; KF – Cephalotin; FEP – Cefepime; CTX – Cefotaxime; FOX – Cefoxitin, CAZ – Ceftazidime; CXM – Cefuroxime; CIP – Ciprofloxacin; C – Choramphenicol; ENR – Enrofloxacin; ETP – Ertapenem; FFC – Florphenicol; FOS - Fosfomycin, CN – Gentamycin; IPM – Imipenem; K – Kanamycin; LEV – Levofloxacin; MEM – Meropenem: S3 - Sulfonamides Compounds; TE – Tetracycline; TOB- Tobramycin; SXT – Sulfonamide+Trimethoprim; W – Trimethoprim.

Strain	TOB			SXT			W		
Susceptibility evaluation	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**
Mm 1	20	S	WT	24	S	NA	14	I	NA
Mm 2	20	S	WT	24	S	NA	18	S	NA
Mm 3	18	S	WT	24	S	NA	16	I	NA
Mm 4	22	S	WT	26	S	NA	18	S	NA
CB/ ECOFF		**S≥17 R<14**	**≥16**		**S≥16 R<13**	**ND**		**S≥18 R<15**	**ND**

IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Classification not available; ND – No data yet

Table 12 - Antimicrobial susceptibility test Results – inhibition Zone Diameter observed in the *K.oxytoca* strains (In milimeters)

Abbreviations: NA – Nalidixic Acid; AK – Amikacin, AML – Amoxicillin, AMC – Amoxicillin+Clavulanic Acid; CL – Cephalexin; KF – Cephalotin; FEP – Cefepime; CTX – Cefotaxime; FOX – Cefoxitin, CAZ – Ceftazidime; CXM – Cefuroxime; CIP – Ciprofloxacin; C – Choramphenicol; ENR – Enrofloxacin; ETP – Ertapenem; FFC – Florphenicol; FOS - Fosfomycin, CN – Gentamycin; IPM – Imipenem; K – Kanamycin; LEV – Levofloxacin; MEM – Meropenem: S3 - Sulfonamides Compounds; TE – Tetracycline; TOB- Tobramycin; SXT – Sulfonamide+Trimethoprim; W – Trimethoprim.
IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Strain		**NA**			**AK**			**AML**			**AMC**			**CL**			**KF**			**FEP**			**CTX**	
	IZD	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**
Ko 1	20	S	NA	20	S	WT	0	R	NWT	26	S	WT	10	R	NA	26	S	NA	26	S	NA	34	S	NA
Ko 2	20	S	NA	18	S	WT	0	R	NWT	24	S	WT	14	S	NA	14	I	NA	24	S	NA	26	S	NA
CB/ ECOFF		**S≥19; R<13**	**ND**		**S≥16 R≤13**	**≥18**		**S≥21 R≤13**	**ND**		**S≥17 R<17**	**≥17**		**S≥14 R<14**			**S≥18 R<14**			**S≥24 R<21**			**S≥20 R<17**	

Strain		**FOX**			**CAZ**			**CXM**			**CIP**			**C**			**ENR**			**ETP**			**FFC**	
	IZD	**CB[b]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**
Ko 1	28	S	WT	32	S	NA	18	S	NA	30	S	NA	28	S	NA	30	S	NA	32	S	NA	28	NA	NA
Ko 2	24	S	WT	28	S	NA	20	S	NA	26	S	NA	26	S	NA	26	S	NA	32	S	NA	28	NA	NA
CB/ ECOFF		**S≥18 R<14**	**≥19**		**S≥22 R<19**	**ND**		**S≥18 R<18**	**ND**		**S≥22 R<19**	**ND**		**S≥17 R<17**	**ND**		**S≥23 R<16**	**ND**		**S≥25 R<22**	**ND**		**ND**	**ND**

Strain		**FOS**			**CN**			**IPM**			**K**			**LEV**			**MEM**			**S3**			**TE**	
Susceptibility evaluation	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**
Ko 1	20	S	NA	22	S	WT	32	S	NA	22	S	NA	28	S	NA	24	S	NWT	0	R	NWT	20	S	NA
Ko 2	18	S	NA	22	S	WT	26	S	NA	20	S	NA	26	S	NA	24	S	NWT	18	S	NA	20	S	NA
CB/ ECOFF		**S≥16 R<13**	**ND**		**S≥17 R<14**	**≥16**		**S≥22 R<16**	**ND**		**S≥18 R<13**	**ND**		**S≥22 R<19**	**ND**		**S≥22 R<16**	**≥25**		**S≥17 R<12**	**ND**		**S≥19 R<13**	**ND**

Abbreviations: NA – Nalidixic Acid; AK – Amikacin, AML – Amoxicillin, AMC – Amoxicillin+Clavulanic Acid; CL – Cephalexin; KF – Cephalotin; FEP – Cefepime; CTX – Cefotaxime; FOX – Cefoxitin, CAZ – Ceftazidime; CXM – Cefuroxime; CIP – Ciprofloxacin; C – Choramphenicol; ENR – Enrofloxacin; ETP – Ertapenem; FFC – Florphenicol; FOS - Fosfomycin, CN – Gentamycin; IPM – Imipenem; K – Kanamycin; LEV – Levofloxacin; MEM – Meropenem: S3 - Sulfonamides Compounds; TE – Tetracycline; TOB- Tobramycin; SXT – Sulfonamide+Trimethoprim; W – Trimethoprim.

Strain		**TOB**			**SXT**			**W**	
Susceptibility evaluation	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF**
Ko 1	18	S	WT	28	S	NA	20	S	WT
Ko 2	6	R	NWT	13	I	NA	9	R	NWT
CB/ ECOFF		**S≥17 R<14**	**≥16**		**S≥16 R<13**	**ND**		**S≥18 R<15**	**≥20**

IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Table 13 -Antimicrobial susceptibility test Results – inhibition Zone Diameter observed in the *Enterococcus faecalis* strains (In milimeters)

Abbreviations: DA – Clindamycin; AMP – Ampicillin, E – Erythromycin, STR – Streptomycin; CN – Gentamycin; C – Chloramphenicol; LZD – Linezolid; LEV – Levofloxacin, TEC – teicoplanin; TE - Tetracycline TOB – Tobramycin; VA - Vancomycin; F – Nitrofurantoin. IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Strain	DA			AMP			E			STR			CN			C			LZD			LEV		
	IZD	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[B]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**
Ef 1	0	R	NWT	24	S	WT	10	R	NA	0	R	NWT	12	I	WT	22	I	NA	34	S	WT	0	R	NWT
Ef 2	0	R	NWT	24	S	WT	12	R	NA	0	R	NWT	12	I	WT	18	I	NA	32	S	WT	0	R	NWT
Ef 3	0	R	NWT	20	S	WT	8	R	NA	0	R	NWT	12	I	WT	24	S	NA	34	S	WT	0	R	NWT
Ef 4	0	R	NWT	20	S	WT	0	R	NWT	0	R	NWT	10	R	WT	24	S	NA	30	S	WT	0	R	NWT
Ef 5	0	R	NWT	24	S	WT	0	R	NWT	0	R	NWT	12	I	WT	20	I	NA	34	S	WT	0	R	NWT
Ef 6	0	R	NWT	24	S	WT	0	R	NWT	0	R	NWT	12	I	WT	24	S	NA	32	S	WT	0	R	NWT
Ef 7	0	R	NWT	24	S	WT	20	I	NA	0	R	NWT	10	R	WT	22	I	NA	30	S	WT	20	NA	NA
Ef 8	0	R	NWT	24	S	WT	0	R	NWT	0	R	NWT	10	R	WT	20	I	NA	24	S	WT	0	R	NWT
CB/ ECOFF		**S≥21 R≤14**	**ND**		**S≥10 R<8**	**≥10**		**S≥23 R<13**	**ND**		**ND**	**ND**		**S≥15 R<12**	**≥8**		**S≥23 R≤12**	**ND**		**S≥19 R<19**	**≥19**		**ND**	**ND**

Abbreviations: DA – Clindamycin; AMP – Ampicillin, E – Erythromycin, STR – Streptomycin; CN – Gentamycin; C – Chloramphenicol; LZD – Linezolid; LEV – Levofloxacin, TEC – teicoplanin; TE - Tetracycline TOB – Tobramycin; VA - Vancomycin; F – Nitrofurantoin. IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Strain	TEC			TE			TOB			VA			F		
	IZD	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[b]**	**ECOFF**	**IZD**	**CB[a]**	**ECOFF[a]**	**IZD**	**CB[a]**	**ECOFF[a]**
Ef 1	20	S	WT	0	R	NWT	10	NA	NA	20	S	WT	26	S	WT
Ef 2	18	S	WT	0	R	NWT	10	NA	NA	18	S	WT	22	S	WT
Ef 3	20	S	WT	0	R	NWT	12	NA	NA	20	S	WT	26	S	WT
Ef 4	18	S	WT	0	R	NWT	10	NA	NA	18	S	WT	22	S	WT
Ef 5	20	S	WT	0	R	NWT	10	NA	NA	20	S	WT	26	S	WT
Ef 6	20	S	WT	0	R	NWT	10	NA	NA	20	S	WT	26	S	WT
Ef 7	20	S	WT	24	S	NA	10	NA	NA	18	S	WT	26	S	WT
Ef 8	18	S	WT	0	R	NWT	10	NA	NA	18	S	WT	22	S	WT
CB/ ECOFF		**S≥16 R<16**	**≥16**		**S≥19 R<15**	**ND**		**ND**	**ND**		**S≥12 R<12**	**≥12**		**S≥15 R<15**	**≥15**

Table 14-Antimicrobial susceptibility test Results – inhibition Zone Diameter observed in the staphylococci strains (In milimeters)

Abbreviations: FD – Fusidic acid, AK – Amikacin; AMC – Amoxicillin+Clavulanic acid; FFC – Florphenicol; CTX – Cefotaxime; P – Penicillin; FOS – Fosfomycin; F – Nitrofurantoin; AMP – Ampicillin; ENR – Enrofloxacin; DA – Clindamycin, E – Erythromycin, TE– Tetracycline, CN – Gentamycin; KF – Cefalotin; OX – Oxacillin; VA – Vancomycin; LZD – Linezolid; SXT – Sulfmethoxazole+Trimethoprim. IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Disks	FD			AK			AMC			FFC			CTX			P			FOS		
Strain	**IZD**	**CBP[a]**	**ECOFF[a]**	**IZD**	**CBP[a]**	**ECOFF[a]**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**
Sa 1	24	S	WT	25	S	WT	30	S	NA	26	NA	NA	28	S	NA	22	R	NWT	34	NA	NA
Sa 2	26	S	WT	22	S	WT	20	S	NA	26	NA	NA	17	I	NA	17	R	NWT	16	NA	NA
CBP/ ECOFF		**S≥24 R<24**	**≥24**		**S≥18 R<16**	**≥18**		**S≥20 R<20**	**ND**		**ND**			**S≥23 R<15**	**ND**		**S≥29 R<29**	**≥29**		**ND**	**ND**
	FD			**AK**			**AMC**			**FFC**			**CTX**			**P**			**FOS**		
Ss	26	S	NA	24	S	NA	40	S	NA	30	NA	NA	36	S	NA	38	S	NA	46	S	NA
Sd	26	S	NA	26	S	NA	44	S	NA	32	NA	NA	40	S	NA	42	S	NA	40	S	NA
S.haem	34	S	NA	16	R	NA	34	S	NA	32	NA	NA	40	S	NA	26	R	NA	48	S	NA
S.hom	0	R	NA	36	S	NA	34	S	NA	38	NA	NA	32	S	NA	23	R	NA	42	S	NA
CBP/ ECOFF		**S≥24 R<24**	**ND**		**S≥22 R<18**	**ND**		**S≥19 R<15**	**ND**		**ND**	**ND**		**S≥23 R<15**	ND		**S≥29 R<29**	**ND**		**S≥14 R<14**	**ND**

Abbreviations: FD – Fusidic acid, AK – Amikacin; AMC – Amoxicillin+Clavulanic acid; FFC – Florphenicol; CTX – Cefotaxime; P – Penicillin; FOS – Fosfomycin; F – Nitrofurantoin; AMP – Ampicillin; ENR – Enrofloxacin; DA – Clindamycin, E – Erythromycin, TE– Tetracycline, CN – Gentamycin; KF – Cefalotin; OX – Oxacillin; VA – Vancomycin; LZD – Linezolid; SXT – Sulfmethoxazole+Trimethoprim. IZD – inhibition zone diameter, CB – clinical breakpoints; ECOFF – epidemiological cut off's. [a] EUCAST parameters;[b] CLSI Vet S1-02 (2013). NA – Not available; ND – No data

Disks		**F**			**AMP**			**ENR**			**DA**			**E**			**TE**			**CN**	
Strain	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[a]**	**ECOFF**	**IZD**	**CBP[a]**	**ECOFF**	**IZD**	**CBP[a]**	**ECOFF[a]**	**IZD**	**CBP[a]**	**ECOFF[a]**
Sa 1	22	S	NA	23	R	NA	32	NA	NA	27	S	NA	0	R	NWT	28	S	WT	22	S	WT
Sa 2	24	S	NA	17	R	NA	32	NA	NA	28	S	NA	0	R	NWT	30	S	WT	21	S	WT
CBP/ ECOFF		**S≥17 R<13**	**ND**		**S≥29 R<29**	**ND**		**ND**	**ND**		**S≥22 R<19**	**≥22**		**S≥21 R<18**	**≥21**		**S≥22 R<19**	**≥22**		**S≥18 R<18**	**≥18**

Disks		**F**			**AMP**			**ENR**			**DA**			**E**			**TE**			**CN**	
Ss	29	S	NA	38	S	NA	34	S	NA	32	S	NA	30	S	NA	34	S	WT	32	S	NA
Sd	30	S	NA	42	S	NA	28	S	NA	34	S	NA	32	S	NA	35	S	WT	28	S	NA
S.haem	28	S	NA	29	S	NA	36	S	NA	36	S	NA	36	S	NA	34	S	WT	34	S	NA
S.hom	26	S	NA	25	S	NA	20	I	NA	36	S	NA	0	R	NWT	26	I	NWT	32	S	NA
CBP/ ECOFF		**S≥17 R<14**	**ND**		**S≥22 R<18**	**ND**		**S≥23 R<16**	**ND**		**S≥22 R<19**	**ND**		**S≥21 R≤18**	**ND**		**S≥29 R<19**	**≥29**		**S≥22 R<22**	**ND**

Disks		**KF**			**OX**			**VA**			**LZD**			**SXT**	
Strain	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[b]**	**ECOFF**	**IZD**	**CBP[a]**	**ECOFF[a]**	**IZD**	**CBP[a]**	**ECOFF[a]**
Sa 1	33	S	NA	21	R	NA	21	NA	NA	32	S	WT	24	S	WT
Sa 2	31	S	NA	20	R	NA	17	NA	NA	28	S	WT	23	S	WT
CBP/ ECOFF		**S≥18 R<14**	**ND**		**S≥22 R<22**	**ND**		**ND**	**ND**		**S≥19 R<19**	**≥19**		**S≥17 R<14**	**≥17**

Disks		KF			OX			VA			LZD			SXT	
Ss	42	S	NA	32	S	NA	20	S	NA	32	S	NA	22	S	NA
Sd	48	S	NA	34	S	NA	20	S	NA	36	S	NA	26	S	NA
S.haem	44	S	NA	26	S	NA	19	S	NA	30	S	NA	30	S	NA
S.hom	40	S	NA	19	R	NA	23	S	NA	42	S	NA	32	S	NA
CBP/ ECOFF		**S≥18 R<14**	**ND**		**S≥20 R<20**	**ND**		**S≥15 R<15**	**ND**		**S≥19 R<19**	**ND**		**S≥17 R<14**	**ND**

Escherichia coli strains in bottlenose dolphins under human care: first report of the O25b:H4-B2-ST131 *E. coli* pathogenic clone

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Objective: To isolate *Escherichia coli* from bottlenose dolphins (*Tursiops truncates*) under human care and characterize its virulence and antimicrobial resistance.

Methods: The blow samples used in this study were collected from the blowhole of bottlenose dolphins as part of an ongoing project on the microbiota of the respiratory tract of bottlenose dolphins at Zoomarine Water Park, Portugal. Nine *E. coli* strains were isolated and classified into phylogenetic groups by PCR. Allele-specific PCR was performed on group B2 isolates to identify the O25-ST131 clone. The presence of 8 Pathogenicity islands (PAIs) markers (I_{J96}, II_{J96}, I_{CFT073}, II_{CFT073} and I–IV_{536}) was assessed by PCR. Susceptibility testing to 27 antimicrobials was performed using the disk diffusion method. Results were interpreted according to clinical breakpoints from CLSI M31-A3 and M100-S22. ESBL and *p*AmpC β-lactamases genes were identified by PCR followed by nucleotide sequencing. The aminoglycoside resistance *aac(6')-Ib* gene was also screened by PCR. *E. coli* clonality was assessed by pulsed-field gel electrophoresis (PFGE) using the PulseNet protocol.

Results: All nine *E. coli* isolates showed resistance to fluoroquinolones and aminoglycosides; 5 were also resistant to 3rd generation cephalosporins (3 positive for $bla_{CTX\text{-}M\text{-}15}$ and 2 for $bla_{CTX\text{-}M\text{-}32}$); 1 isolate harboured the $bla_{DHA\text{-}1}$ gene and 3 isolates harboured the *aac(6')-Ib* gene. Seven isolates were positive for clonal group O25b:H4-B2-ST131 and the remaining 2 isolates were classified as non-virulent phylogroup B1. All the ST131 *E. coli* strains were positive for PAI IV_{536}, PAI I_{CFT073} and PAI II_{CFT073}, as shown on Table 1. PFGE analysis showed 2 clusters: cluster 1 included all ST131 strains with 86.6 % similarity and cluster 2 showed the two B1 strains with 91.4% similarity (Figure 1). The two clusters have 71.2% similarity between them.

Conclusion: Colonization of the respiratory tract of bottlenose dolphins with *E. coli* has been previously reported, however to our knowledge this is the first detection of the O25b:H4-ST131 human pandemic virulent clone in dolphins under human care. We therefore describe that dolphins under human care act has a reservoir of the O25b:H4-ST131 virulent clone.