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TOPICAL AZITHROMYCIN APPLICATION AND ITS INFLUENCE ON *PSEUDOMONAS AERUGINOSA* N-ACYL HOMOSERINE LACTONES ASSOCIATED WITH CANINE OTITIS

PhD Thesis

VPLIV LOKALNEGA ZDRAVLJENJA Z AZITROMICINOM NA IZLOČANJE N-ACYL HOMOSERIN LAKTONOV V KLINIČNIH VZORCIH PSOV Z VNETJEM UŠES, POVZROČENIM Z BAKTERIJO *PSEUDOMONAS AERUGINOSA*

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TOPICAL AZITHROMYCIN APPLICATION AND ITS INFLUENCE ON *PSEUDOMONAS AERUGINOSA* N-ACYL HOMOSERINE LACTONES ASSOCIATED WITH CANINE OTITIS

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The doctoral thesis is the result of my own research work.

Javid Hosseini Tabrizi

This thesis is dedicated to my parents

for their endless love, support and encouragement

To my dear mentors

Dr. Tina Kotnik, assistant professor

Dr. Modest Vengušt, assistant professor

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ABBREVIATIONS

AHLs	N acyl homoserine lactones
AI-1	auto-inducer 1
AIPs	Auto-(3) inducing polypeptides
AIS	Autoinducers
ALT	Alanine aminotransferase
AP	Alkaline Phosphatase
BHL	N-butyryl-L-homoserine lactone (C4-HSL)
CBC	Complete blood count
dHL	N-decanoyl-L- homoserine lactone
EHEC	Enterohaemorrhagic *Eschericia coli*
HBHL	N-(3-hydroxy-butyryl)-L- homoserine lactone
HHL	N-hexanoyl-L- homoserine lactone (C6-HSL)
HUS	haemolytic uremic syndrome
LB	Luria Bertani medium
MIC	Minimum inhibitory concentration
OHL	N-octanoyl-L- homoserine lactone
OdDHL	N-(3-oxo-dodecanoyl)-L-homoserine-lactone (C12-HSL)

Ps	Primary sample
PQS	*Pseudomonas* Quorum sensing
QS	Quorum sensing
QSM	Quorum-sensing molecule
SID	Medication once a day
TTP	Thrombotic thrombocytopenic purpura
TLC	Thin layer chromatography
CSP	Competence signal peptides

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ABSTRACT

Key words**:** otitis externa-drug therapy – microbiology; *Pseudomonas* infections – drug therapy; *Pseudomonas aeruginosa* – pathogenicity; virulence factors; antibacterial agents – pharmacology; azithromycin; quorum sensing – drug effects; acyl – butyrolactones – metabolism; gene expression regulation, bacterial; dogs

The aim of our study was to evaluate whether *P. aeruginosa* produces N-acyl homoserine lactones (AHLs) in otitis externa of dogs and to evaluate whether topically administered azithromycin could influence the production of N-acyl homoserine lactones (AHLs) that would consequently influence the quorum sensing (QS) phenomenon. A hundred and thirty-five ear-lavage samples were collected from 21 dogs with *P. aeruginosa*-associated otitis. Treatment protocol was designed based on the clinical observation as well as on the cytological and parasitological examination. Dogs were evaluated on days 0, 7, 14, 21, 28, 42 and 56. Clinical signs were evaluated and scored. Dogs were randomly divided into two groups; dogs from one group were treated with Tris-EDTA/chlorhexidine solution (Otodine^{R}) and azitromycine while dogs from the other group were treated topically with Tris-EDTA/chlorhexidine solution and placebo. Concentrations of three main AHLs, C4-HSL(BHL), C6-HSL (HHL) and 3-oxo-c12-HSL (OdDHL) were analyzed in the ear-lavage samples to assess their influence on other selected variables. Time had a significant effect on all clinical signs individually and it also had a significant effect on overall clinical signs. Also, time had a significant effect on the reduction of rod-shaped bacteria in the ear canal. A significant association of BHL with rod-shaped bacteria was found indicating that a BHL increase by one unit corresponded to a decrease of 0.2 rod-shaped bacteria /OIF. A significant association of HHL with rod-shaped bacteria was also found indicating that a HHL increase by one unit corresponded to a decrease of 5.2 rod-shaped bacteria/OIF. Treatment with azithromycin, methylprednisolone, fluoroquinolone antibiotics or time had no effect on the presence of OdDHL in ear canal samples. This study has shown that *P. aeruginosa* has expressed its virulence factors in dogs with otitis *via* the QS phenomenon. The detection of *P. aeruginosa*-specific AHLs in high concentrations showed this bacterium-produced AHLs in the case of otitis externa.

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IZVLEČEK

Ključne besede**:** otitis zunanjega ušesa – zdravljenje z zdravili – mikrobiologija; pseudomonasna okužba – zdravljenje z zdravili; *Pseudomonas aeruginosa* – patogenost; dejavniki virulence; antibakterijska sredstva – farmakologija; azitromicin; zaznavanje celične gostote – učinki zdravil; acil – butirolaktoni – metabolizem; gensko izražanje, bakterijska regulacija; psi

Namen naše raziskave je bil ugotoviti, ali bakterije vrste *P. aeruginosa* med okužbo zunanjih sluhovodov psov proizvajo N-acyl homoserin laktone (AHL) in tudi ali lokalno zdravljenje z azitromicinom lahko vpliva na proizvodnjo molekul AHL, kar bi posledično vplivalo na fenomen quorum sensing (QS). Enaindvajsetim psom z vnetjem zunanjega sluhovoda, povzročenega z bakterijo *P. aeruginosa,* smo odvzeli 135 izpirkov zunanjih sluhovodov. Protokol obravnave je zajemal klinični pregled, citološke, parazitološke in hematološke preiskave. Vzorčili smo naslednje dni v obdobju dveh mesecev: 0., 7., 14., 21., 28., 42. in 56. dan. Klinični znaki vnetja so bili ovrednoteni s številko od 0 do 4 glede na izraženost. Pse smo dvojno slepo razdelili v dve skupini: psi prve skupine so bili zdravljeni z antiseptično raztopino Tris-EDTA/klorheksidin (Otodine^{R}) in azitromicinom, psi druge skupine pa so bili lokalno zdravljeni z antiseptično raztopino Tris-EDTA/ klorheksidin (Otodine^{R}) in placebom (fiziološka raztopina). V izpirkih zunanjega sluhovoda smo merili koncentracije treh vrst molekul AHL, C4-HSL(BHL), C6-HSL (HHL) in 3-oxo-c12-HSL (OdDHL) ter statistično vrednotili njihov vpliv na druge izbrane spremenljivke. Čas je statistično značilno vplival na vsakega od kliničnih parametrov posamezno kot tudi na skupni klinični parameter. Čas je imel statistično značilen vpliv na zmanjšanje števila paličastih bakterij v zunanjih sluhovodih. Dokazali smo statistično značilno povezavo BHL-jev s paličastimi bakterijami. Porast koncentracije molekul BHL za eno enoto je bil vzročno povezan z zmanjšanjem 0,2 (±0,08) paličastih bakterij v velikem vidnem polju mikroskopa (1000-kratna povečava). Dokazali smo statistično značilno povezavo HHL-jev s paličastimi bakterijami. Porast koncentracije molekul HHL za eno enoto je bil vzročno povezan z zmanjšanjem 5,2 (±0,08) paličastih bakterij v velikem vidnem polju mikroskopa (1000x povečava). Zdravljenje z azitromicinom, metilprednizolonom in fluorokinoloni ni vplivalo na prisotnost OdDHL v sluhovodih psov, nanje ni vplival niti čas. Dokazali smo, da bakterija *P. aeruginosa* proizvaja molekule AHL pri okužbi zunanjega sluhovoda psov in ureja faktorje virulence pri vnetju zunanjega sluhovoda psov s pomočjo pojava QS.

1 INTRODUCTION

Canine otitis and ear infection describe two different conditions. Otitis refers to an inflammation-within ears and may occur due to a variety of different reasons including food allergies, atopy, entrance of a foreign body, etc. On the other hand, an ear infection refers to an actual infection of the ear with bacteria, yeasts or parasites. In most cases, an infection cannot occur without a pre-existing inflammation of ear tissue provoked by one of the so-called primary factors (1). Diagnosis is made by an examination of ears and also an overall examination of the dog. Swabs of debris from dogs' ears may be examined microscopically for evidence of ear parasites. Ear cytology is done on preparations of ear canal swabs to evaluate abnormal cell types and the presence of yeasts and/or bacteria (2). If a bacterial infection is found, it may be necessary to identify from the culture of ear swab the specific type of microorganism present and to determine which antibiotic would be most effective to control the infection (1). *Pseudomonas aeruginosa* (*P. aeruginosa*) is the most common bacterium associated with chronic otitis externa and otitis media in dogs. Ear canals infected with *P. aeruginosa* are often ulcerated and filled with purulent exudate. Cytology of purulent exudates from dogs with *Pseudomona*s otitis will show numerous rod-shaped bacteria and inflammatory cells. *Pseudomona*s may be present in pure culture or accompanied with cocci, other rods, and/or *Malasezzia* (3). Otoscopic examination of the ear canal is an important part of the procedure to assess the clinical state of the ear canal and tympanic membrane. Since ears infected with *Pseudomonas* otitis are usually quite painful, general anesthesia is sometimes recommended (4). If an ear canal is too stenotic, a decision may be made to delay this procedure for 2 weeks starting topical treatment and topical or systemic steroids in the interim (5). *P. aeruginosa* is typically multiple-drug resistant bacterium which can be determined by the culture and susceptibility test. This can be attributed to a combination of plasmid-mediated mutations and decreased antibiotic penetration. Various studies describe *in vitro* susceptibility of *P. aeruginosa* (6).

Pathogens use various mechanisms to change pathogenicity. One of these mechanisms is quorum sensing (QS). In accordance with a simple explanation, QS occurs when individual bacteria in the population may find advantages in acting together and making collective decisions. They start producing the so-called signal molecules and when the concentration of these molecules in the microenvironment exceeds a certain threshold, bacterial population starts acting as a single organism, collectively expressing virulence genes, biofilm-forming genes and therefore causing classic treatment methods to become ineffective. Recent studies have shown that QS causes tolerance to various antimicrobial treatments and immune modulation (7). Regulation of virulence via QS confers a strategy advantage over host defenses. Consequently, a drug capable of blocking QS is likely to increase susceptibility of the infecting organism to host defenses and its clearance from the host. Recent use of QS signal blockers to attenuate bacterial growth and virulence is considered a new strategy for treatment of resistant bacterial pathogens (8). One of these blockers is azithromycin, an azalide, a subclass of macrolide antibiotics. This antibiotic could impede QS and reduce pathogenicity of *P. aeruginosa* (9).

The aim of this study was to evaluate whether *P. aeruginosa* produces N-acyl homoserine lactones (AHLs) in otitis externa of dogs and to evaluate whether topically administered azithromycin could influence the production of AHLs that would consequently influence the QS phenomenon.

1.1. Anatomy of canine external ear canal

An ear is appropriately called a *vestibulocochlear* organ since it not only enables animals to hear but also provides them with a sense of balance. The external portion of the ear consists of a flap known as pinna and an opening at the base of the ear. At the external orifice of the ear, auricular cartilage begins to roll into a funnel shape that extends downwards forming a vertical ear canal, then turns inwards forming a horizontal ear canal which terminates with the tympanic membrane or eardrum (10). Lumen diameter is approximately 0.5 to 1 cm. The horizontal part of the external ear canal is lined by specialized skin (about 1mm thick) which is rich in sebaceous glands and cerumen-producing glands. Cerumen is a complex mixture of exfoliated cells, waxes, oils, free fatty acids, esters, immunoglobins such as IgA, IgG, IgM and proteins which act as an antimicrobial and protective barrier for the ear canal (11). The main function of the external ear canal and pinna is to collect sound waves and transmit them to the tympanic membrane. Mechanical stimuli produced by sound waves are transmitted from the tympanic membrane to the inner ear by auditory ossicles (malleus, incus and stapes). In addition to the auditory ossicles, the Boney chamber of the middle ear (bulla) is lined by the mucus membrane and not skin, and it contains ligaments, muscles, nerves (chorda tympani) and the auditory tube. Mechanical stimuli transmitted by auditory ossicles are finally transformed into nerve impulses in the *cochlea.* The action of small amounts of fluid and microscopic crystals on neuroreceptors within the vestibule provides the animal with a perception of altitude and movement of its head with respect to gravity (10). Both functions are performed in the inner ear. The inner ear is housed in the petrosal temporal bone. The petrous portion of the temporal bone or pyramid is pyramidal and is wedged in at the base of the skull between the sphenoid and occipital bones. A portion of the petrosal bone is in close proximity to the cerebrum and another portion is juxtaposed to the cerebellum. In the middle part of the cerebella portion of the petrosal bone, the internal auditory meatus (*meatus acusticus internus*) is located. It carries nerves from inside the

cranium towards the middle and inner ear compartments, namely the cranial nerve VII (facial nerve) and the cranial nerve VIII (auditory vestibular nerve) (10). Young dogs can detect air conducted stimuli ranging between 0 to 10 decibels (dB) and bone conducted stimuli of about 50 to 60 dB (1).

As already mentioned, external ear canals are lined by skin. Disorders that affect the skin (like an allergy) will also affect the integrity, function and protecting mechanisms of the skin in the ear canal. The result of this imbalance can predispose to *otitis externa*, altering the integrity and defense mechanisms of the skin, increasing the volume and altering the composition of cerumenous gland secretions (1,12).

1.2. Otitis Externa

1.3. Otitis Externa in dogs

By definition, otitis externa is an inflammation of the ear canal. Dogs with floppy ears seem more prone since air flow is limited and a warm, moist environment builts up which may be conductive to infection. Infections usually result from a pre-existing inflammation or they are provoked by improper grooming techniques. Infections can be both, of bacterial and fungal origin. Parasitic infestations can provoke otitis as a primary cause. *Malassezia pachydermatis* is yeast, commonly involved in otitis externa of dogs. *Staphylococcus pseudintermedius* (*S. pseudintermedius*) is the most common bacterial organism. The most serious ear infection with pus in the ear may be caused by *P. aeruginosa* (1,13).

Otitis externa is a common condition in dogs accounting perhaps for 15 % of all dogs presented for veterinary care. The true incidence of otitis externa is not known and one study noted that the prevalence of otitis externa in dogs is 10 - 20 % and perhaps as high as 30 - 40 % in tropical and subtropical environments (14). Another study has noticed that 26 % of the otitis cases are parasitic, 32 % allergic, 8 % bacterial and 2 % are tumor,

metabolic, autoimmune and cornification disorders (15). Based on researches, incidence for *Pseudomonas* otitis is about 1.8 % (16).

1.3.1. *Predisposing factors for Otitis externa*

Predisposing factors increase the risk of developing the disease in combination with primary, secondary and perpetuating causes and the result is a clinical disease. The way to successfully manage and treat otitis externa is by recognizing and controlling these factors (1,13). There have been a lot of predisposing factors recognized, but the most common among them are connected to conformation (like hairy concave pinna, pendulous pinna or stenotic ear canal), excessive moisture in the ear canal or systemic diseases that provoke immunosuppresion.

1.3.2. *Primary factors of otitis externa*

Many skin diseases may act as the primary trigger for otitis and subsequent *Pseudomonas* infection (17). This group of causes directly induces otitis externa. The most common amongst them are atopic dermatitis, food hypersensitivity, foreign bodies, keratinization disorders and ear mites (1,13). Allergic dermatitis can initiate as many as 43 % of otitis externa cases by changing the balance of physiological secretions and microflora of the ear canal resulting in opportunistic infections (18). *Otodectes cynotis* is one of the most common ear mites involved in 5 - 10 % of otitis externa in dogs. Mites can cause hypersensitivity reactions (the Arthus type and immediate type) as a factor for initiating an inflammation of the ear canal and secondary infections (13). Atopic dermatitis (involved in 83 % of cases), food hypersensitivity and contact dermatitis can cause otitis externa by inducing a chronic inflammation and leading to a secondary bacterial or yeast infection. In young dogs of less than 6 months of age having acute bilateral otitis without the presence of foreign bodies or ear mites, food hypersensitivity should be considered. Contact dermatitis may be caused by medications and chemicals (for example neomycin and propylene glycol) (13).

1.3.3. *Secondary causes of otitis externa*

Secondary factors can produce otitis only in the abnormal ear or in combination with predisposing factors. This means that these organisms or agents may also be found in a normal ear without producing the disease. Elimination of the concurrent predisposing factor or a primary disease is therefore very important in managing otitis (1). Amongst secondary factors, the most important are bacteria including *S. pseudintermedius* and *P. aeruginosa* that are involved in the majority of chronic otitis cases (19), these two species being isolated from more than 70 % of all cases (20). Amongst yeasts, *Malassezia pachydermatis* is the most common and may be found in as many as 36 % of normal canine ears. It may be involved in as many as 76 % of otitis externa cases frequently in combination with *Staphylococcus* sp. Scarification, moisture and alkalinisation can predispose to *P. aeruginosa* ear infection in normal dogs. After bacteria infect the ear tissue, they contribute to the inflammation, damage and clinical signs (1,13).

1.3.4. *Perpetuating factors*

Perpetuating factors prevent resolution of otitis externa or otitis media. They result from an inflammation and pathologic responses of skin and otic structures. In chronic cases, one or more of these factors are present. Perpetuating factors may be the major reason for poor response to therapy regardless of predisposing factors and primary causes present (13). Amongst more common, there is altered epithelial cell migration in epidermis of the external ear canal, epithelial folds and stenosis of the ear canal.

1.4. Otitis Media

Otitis media is an inflammation of the middle ear. A normal middle ear cavity may contain some bacteria (staphylococci, streptococci) and yeasts without exudates production or inflammatory cells recruitment. The presence of exudates within the

tympanic cavity is difficult to treat with topical therapy and often remains a source of infection and proinflammatory toxins and debris to reach the external ear canal (1).

1.5. Clinical signs of otitis

The most common symptom of otitis externa is aural pruritus or head shaking. As otitis externa progresses, a mild to marked exudates or malodor may develop. Hearing loss may also occur. Physical findings indicative of otitis externa include erythema, swelling, scaling, crusting, alopecia, broken hairs, head shyness, otic discharge (otorrhea), malodor and pain on palpation of the auricular cartilage. Some animals attempt to scratch the ear with the ipsilateral hind paw or shake the head during or after the palpation of the ear canal. Clinical signs of otitis media are quite variable and not specific. Most commonly the symptoms mimic or occur because of concurrent otitis externa. Palpation of the external ear canal and tympanic bulla may provide additional information. Thickness, firmness, and pliability of the vertical and horizontal canal should be determined. Ulceration of the external orifice and/or ear canal should make one consider secondary causes such as *Pseudomonas* infection, *Candida* infection, and topical reactions (1).

1.6. Diagnosis of otitis

Diagnosis of otitis externa is made easily from the history and physical examination. Otoscopic examination is used to detect foreign bodies, to determine whether otitis media is present and to assess what type of lesions, exudates and progressive pathologic changes have occurred. Since dogs with *Pseudomonas* otitis usually have a painful and stenotic ear canal, a decision might be made to delay this procedure. Oral methylprednisolone can be used as a potent anti-inflammatory agent (4) that inhibits the synthesis of almost all known cytokines and cell surface molecules required for immune function (5). Otitis media is much more difficult to diagnose because many patients present with symptoms of only otitis externa. Evidence of inflammation of the tissue surrounding the middle ear or the inner ear usually indicates that otitis media has occurred. Even with an otoscopic

examination, many cases of otitis media may not be detected and, in cases with apparently intact diseased tympanic membranes, otitis media may be present.

1.6.1. *Cytology examination*

Cytology examination of discharge does not usually establish a definitive diagnosis, but it is valuable in determining what infectious agents, if any, are present. Cytology may reveal cocci (especially from genus *Staphylococcus* and *Streptococcus*), rods (especially from genus *Pseudomonas and Proteus*), other gram-positive or gram-negative organisms, budding yeasts (*Malassezia* and *Candida*) or mixed infections. Presence of white blood cells, as well as phagocytosis of bacteria, indicates that the body is responding to the infection and that treatment of infection is warranted (21). For the purpose of cytology evaluation, swab specimen should be rolled onto a glass microscope slide and stained after heat fixing by using a modified Wright's Stainning like Diff-Quik (21). Some studies have shown that the direct microscopic examination has less sensitivity than culture and it may thus give false negative results. One may find negative results on the direct examination, but positive results after culture. The direct examination gives an immediate diagnosis, but culture is necessary for more accurate results. In addition, culture together with biochemical tests offers the possibility of a definitive identification (22).

1.6.2. *Culture and susceptibility testing*

Primary indication for culture and susceptibility testing is the presence of otitis media or severe otitis externa associated with rod-like bacteria when systemic therapy is going to be prescribed. It is common for otitis patients to have multiple types of bacteria isolated from an inflamed ear canal. It is important to remember that resistance to particular antibiotic *in vitro* may not correlate with the clinical response because direct application of medication to the ear canal results in a higher antibiotic concentration than with systemic medication. For some canine otic isolates such as *P. aeruginosa*, β-haemolytic streptococci and enterococci, disk diffusion techniques (Kirby-Bauer) indicate *in vitro*

susceptibility, whereas the minimum inhibitory concentration (MIC) testing indicates resistence (1). The standard method for antimicrobial susceptibility testing is the dilution method which is quantitative in nature. The Kirby-Bauer method has been extrapolated from the dilution method as a less complex mean to determine antimicrobial susceptibility and is currently the most commonly utilized methodology. Compared to the qualitative Kirby-Bauer method, the micro dilution method (MIC) susceptibility testing is considered more reliable and useful for determining optimal therapeutic dosing regimens (16).

1.7. Quorum sensing

Quorum sensing (QS) is a bacterial intercommunication system that controls the expression of multiple genes in response to population density (23). Many bacteria regulate their phenotype via intercellular communication based on the production and collective response to diffusible signal molecules termed autoinducers (24). Under certain circumstances, individuals of the bacterial population may find advantages in acting together and making ‘‘collective decisions’’. This phenomenon is better known as QS. When the concentration of signal molecules produced by surrounding bacteria exceeds a certain threshold, bacterial population acts as a single organism collectively expressing certain genes (7). After the critical threshold of autoinducer concentration is achieved in the bacterial population niche, gene expression and consequently behavior is expressed as biofilm formation (25), expression of virulence determinants (26), production of antimicrobial substances (27), motility (28) and sporulation (29). Targeting of the QS mechanisms may provide a novel strategy for combating bacterial infections in human medicine and in veterinary medicine as well. QS utilizes small, hormone-like molecules called autoinducers (AIs) to monitor the environment. A typical response to an AI involves modulation of gene expression. Multiple QS systems including luxS, AI-2 and AI-3 systems are produced by other enterobacteria such as *Escherichia coli* (both commensal and pathogenic), *Shigella sp., Salmonella sp., Klebsiella pneumoniae* and *Enterobacter cloacae* as well as microbial intestinal flora cultured from stools of healthy human volunteers (7).

Due to the involvement of bacterial QS in pathologically relevant events such as symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation, Qs inhibitors have the potential of being used in antimicrobial therapy as adjuvant (1).

The interest of this doctoral thesis is QS in Gram-negative bacteria. Gram-positive bacteria, however, also employ QS for their interaction within the population and with their environment. They mainly use modified oligopeptides as QS signal molecules (30).

1.7.1. *Quorum sensing in Gram-negative bacteria: N-acyl homoserine lactones*

In Gram-negative bacteria, signal molecules are N-acyl homoserine lactones (AHLs), which are also called auto-inducers 1 (AI-1) (31). They consist of a conserved homoserine lactone ring which is connected to an acyl side chain of a variable length (4–18 carbon atoms) and a variable extra modification. AHLs with a short acyl side chain can diffuse passively in and out of the bacterial cell. LuxI homologous proteins play a major role in the synthesis of the AHLs, which increase in concentration as the cell density increases. At a critical concentration, LuxR homologues bind signal molecules and subsequently regulate gene transcription (7). By synthesis of the analogs of *N*-3-oxododecanoyl-homoserine lactone, the induction of apoptosis in macrophages has been shown. It has revealed that the position of the oxo group in the acyl side chain is crucial for the apoptosis-inducing activity in addition to the presence of the L-homoserine lactone unit. Furthermore, long acyl side chains with hydrophobic distal ends are preferable for the activity (32). Modulation of the physiological processes controlled by acyl HSLs (and, indeed, many of the non-acyl HSL-mediated systems) occurs in a cell density- and growth phase-dependent manner (33). QS-negative mutants of *P. aeruginosa* display reduced virulence in comparison with the QS-proficient parental strains suggesting that QS may give the bacterium a competitive advantage in the pathogenic interaction with the host (34, 35).

1.7.2. *Quorum sensing in Gram-positive and Gram-negative bacteria: auto-inducer 2*

In the late 1990s, the second QS system was discovered in various Gram-negative bacteria. In this system, the auto-inducer (AI-2) is a furanosyl borate ester that is formed by the LuxS protein. To date, AI-2 has been described in more than 50 different bacterial species comprising both Gram-positive and Gram-negative bacteria (7,36). A key step in the AI-2 synthesis is catalyzed by a highly conserved enzyme LuxS. The *luxS* gene is found in a vast number of bacterial species implicating its importance for the basic biological function (23,26). AI-2 is derived from the precursor compound 4,5-dihydroxy-2,3-pentanedione (DPD). LuxS converts S ribosyl homocysteine to homocysteine and DPD. Once outside the cell, DPD can undergo a number of spontaneous chemical rearrangements to form different furans, including the two known AI-2 structures. AI-2 is perceived by the cell in different manners depending on the system. In the *Vibrio harveyi* system, AI- 2 is recognized at the cell surface by the cell membrane-associated receptor LuxP (37). *P. aeruginosa* possesses the third LuxR-type regulator QscR, which lacks a cognate synthase. QscR appears to delay the activation of several quorum-controlled genes (38), possibly by forming heterodimers with LasR and RhlR (39). Considerable evidence has been accumulated for QS being instrumental for virulence: many of the extracellular virulence factors of *P. aeruginosa* are positively controlled by QS (40,41,42).

1.7.3. *QS signals production in enteric bacterial species: Auto-inducer 3*

Enterohaemorrhagic *Eschericia coli* (EHEC) is the cause of various foodborne outbreaks of severe intestinal diseases and the causative agent of the haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans. EHEC motility as well as attaching and effacing virulence genes are regulated by a QS mechanism that involves the poorly characterized auto inducer 3 (AI-3), which is produced by several

enteric bacterial species (7). Since epinephrine/norepinephrine induce the same virulence gene expression in EHEC and since the effects of AI-3 can be inhibited by adrenergic receptor antagonists, AI-3 is probably structurally similar to epinephrine/norepinephrine. In addition, the presence of epinephrine/norepinephrine itself may serve as a QS signal (43).

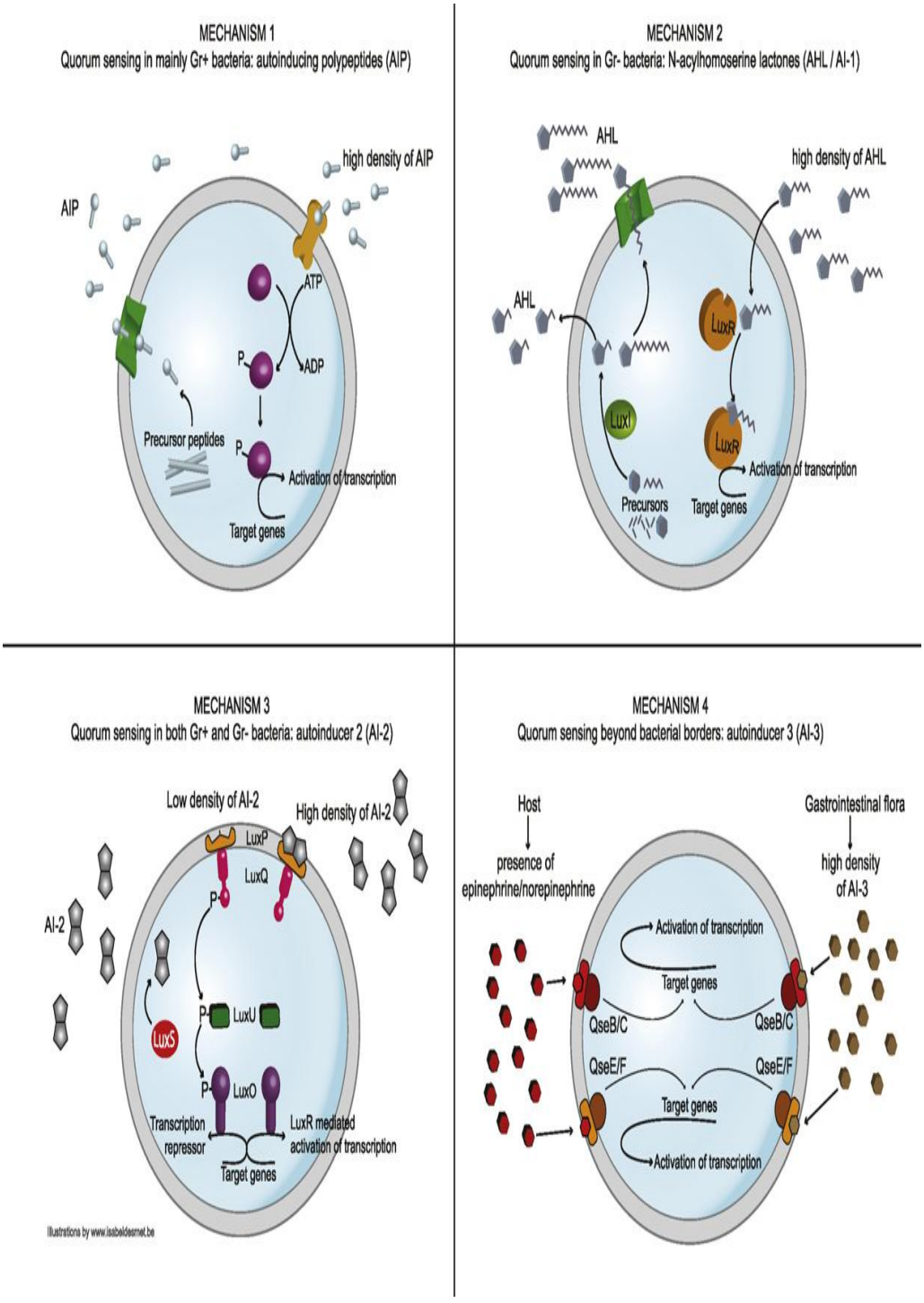


Figure 1: Mechanisms of *quorum sensing*

Courtesy of Filip Boyen (7).

1.7.4. *Acyl Homoserine Lactones (AHLs)*

Acyl homoserine lactone of the QS system has first been described in *Vibrio fischeri* (44). *V. fischeri* is a marine species that can bioluminesce in the light organs of various marine animals such as the Hawaiian bobtail squid *Euprymna scolopes* (45). *V. fischeri* was found to bioluminesce at high cell densities in the liquid batch culture (44) due to an accumulation of the AHL signal 3-oxo-hexanoyl homoserine lactone (46,47). AHLs of different species differ in the acyl side chain, which usually contains between 4 and 14 carbons and which can have an oxo or a hydroxyl substitution at the third position (48,49). AHL signals consist of a homoserine lactone moiety that is linked to an acyl side chain by an amide bond. The acyl side-chain lengths and the degree of substitution can vary from one QS system to another. AHLs can exhibit a wide range of diffusion characteristics. Short side-chain AHLs diffuse freely across cell membranes as long side-chain AHLs may participate to the membrane requiring active efflux for signal export (50).

AHL synthesis is primarily catalyzed by a single enzyme belonging to the LuxI family, while signals are perceived by cytoplasmic DNA-binding regulatory proteins belonging to the LuxR family (51). Competence signal peptides (CSP) are examples of QS molecules that are frequently used by streptococcal species. An accumulation of CSP induces autolysis releasing chromosomal DNA into the environment (52). Subsequent uptake of DNA by neighboring cells is thought to promote horizontal gene transfer (53). Natural competence is therefore thought to be advantageous as a form of group behavior, but CSPs and other peptide-based QS molecules are also implicated to regulate other group-associated behaviors in different Gram-positive species such as biofilm formation (54) and bacteriocin production (55).

At the threshold cell density or AHL concentration in their environment, these AHLs bind and activate their respective regulatory proteins LasR and RhlR. These regulatory proteins bind to the AHL to induce the expression of genes that are under the transcriptional control of the promoters P*rhlI* and P*lasI*, i.e. the regulatory genes *lasI* and *rhlI* and other target genes. These events lead to the expression of LasI and RhlI in a positive feed-back

loop and other factors responsible for virulence and biofilm formation. Gene sequences of these as well as of other QS regulatory systems have been cloned and inserted into plasmid reporter vectors (35). AHL biosynthesis is intimately linked to methionine and S-adenosyl-methionine (SAM) metabolism. In the LasI- and RhlI-catalyzed reactions, the homoserine lactone ring of AHLs originates from SAM and the acyl side chains are derived from fatty acids loaded onto acyl carrier protein (ACP) (56,57). Methylthioadenosine (MTA) is the product of these reactions and an inhibitor of the RhlI enzyme (57). SAM is also a precursor of the polyamine spermidine, with concomitant production of MTA, and a methyl donor in many vitally important reactions with the accompanying formation of S-adenosyl-homocysteine (SAH). *P. aeruginosa*, like other bacteria, has recycling pathways for MTA and SAH. MTA is transformed into methylthioribose- 1-phosphate (MTR-1-P) and adenine by the MtnP phosphorylase. Methionine is regenerated from MTR-1-P via a series of five reactions (58).

1.7.5. *The Agr system as a receptor for AHLs*

Summary of the *Agr* system consists of a ≈3 kb locus containing divergent transcription units driven by P2 and P3 promoters. The P2 operon encodes a two-component system and its auto inducing ligand. The primary function of the operon is to activate the two *agr* promoters. The P3 transcript, RNA III, rather than the response regulator Agr A, is the intracellular effector of target gene regulation. As *agr* is autoinduced by an extracellular ligand, it represents a sensor of population density. Because the activating ligand is encoded within the operon, the circuit is doubly autocatalytic resulting in a very rapid burst of activity once the auto induction threshold has been reached. The expression of this system entails a tremendous metabolic burden resulting in frequent spontaneous *agr* mutants in the laboratory. The *agr*-activating ligand is a post-translationally modified peptide, seven to nine amino acyl residues in length, which is processed from a propeptide encoded by *agrD*. The AIP binds to the N-terminal transmembrane domain of the *agr* signal receptor, *agrC*, activating the *agr* TCS of which AgrA is the response regulator. Activated AgrA then upregulates the P2 and P3 promoters (59).

1.7.6. *Metabolism of N-Acyl homoserine lactones*

Acyl homoserine lactones, in particular those having short acyl chains, are susceptible to spontaneous and reversible, nonenzymatic lactonolysis at pH values above 7.5 (60,61). Moreover, 3-oxo-C12-HSL can slowly rearrange at pH 7.4 to give 3-(1-hydroxydecyclidene)- 5-(2-hydroxyethyl)-pyrrolidine-2,4-dione, a tetramic acid which has antibacterial activity and chelates Fe3+ with high affinity (62). The physiological role of this tetramic acid is unknown. AHLs with acyl side chains of at least 8 carbons can sustain growth of *Pseudomonas spp*. to some extent (63). In *P. aeruginosa*, the enzymatic degradation of AHLs involves at least two AHL acylases, namely PvdQ (63) and Quip, which produce homoserine lactone. A homoserine lactone-lactonase which has not been identified biochemically can then generate homoserine (64). AHLs-degradation enzymes are commonly found in AHL-producers as adequate turnover of signal molecules is an essential feature of QS regulation (65). In *P. aeruginosa*, expression of the acylase-encoding gene pvdQ, which is involved in pigment pyoverdin biosynthesis (66), is positively controlled by LasR in the late exponential phase (67). Balance of production vs. degradation of AHLs is generally in favour of production during growth, but later tips to degradation in the stationary phase (63,68).

1.8. Pseudomonas aeruginosa and quorum sensing

P. aeruginosa infections are challenging to manage due to the bacterium's potential multidrug resistence and the ability to produce several extracellular factors which are involved in the expression of virulence (24). The ability of *P. aeruginosa* to form biofilm and harmonize its virulence via QS is considered important and enables *Pseudomonas* infections to become chronic (69). Since the discovery of cell-to-cell signaling in *P. aeruginosa* in the early 1990s (70), the list of genes reported to be controlled by QS has increased steadily. The first global approach to identifying a larger set of quorum-controlled genes was taken by Whiteley (67). Employment of the QS system is efficient in

a spatially limited environment where *P. aeruginosa* can grow to high densities. Therefore, the long and narrow external ear canal of dogs is a very convenient niche for *P. aeruginosa* colonization and QS expression (71).

P. aeuginosa is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility and an opportunistic human and animal pathogen. It is also an opportunistic pathogen of plants (72). *P. aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), fluorescein (yellow-green and fluorescent, now also known as pyoverdin) and pyorubin (red-brown). *P. aeruginosa* is often preliminarily identified by its pearlescent appearance and grape-like or tortilla-like odour *in-vitro*. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of both pyocyanin and fluorescein as well as its ability to grow at 42° C (73). Although classified as an aerobic organism, *P. aeruginosa* is considered facultative anaerobe by many as it is well adapted to proliferation in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate as a terminal electron acceptor, and, in its absence, it is also able to ferment arginine by substrate-level phosphorylation (74).

The bacterium is catalase+, oxidase+, nitrase+, and lipase+. When grown on TSI medium, it has a K/K/g-/H_2S- profile, meaning that the medium will not change color. Finally, serology based on H & O antigens could help. *P. aeruginosa* is able to swim in liquid by means of flagella and to move on surfaces by means of type IV pili (75). The surface-associated so-called twitching motility is powered by extension and retraction of type IV pili (76). Using the microtitre plate assay, it was showon that flagella or flagellum-driven motility is required for biofilm formation by *P. aeruginosa* and that type IV pili are required for biofilm and microcolony formation by this organism. It was speculated that swimming motility might enable the bacteria to overcome repulsive forces at the surface–water interface so that they reach the surface and that the microcolonies may be formed by twitching motility-driven cell aggregation (77). *P. aeruginosa* biofilm development may be dependent on the carbon source used to support growth and this finding showed that type IV pili are not always necessary for *P. aeruginosa* biofilm or microcolony formation

(77). Transcription of the *P. aeruginosa* LasA and LasB elastase genes (*lasA* and *lasB*, respectively) is activated in a QS, cell density-dependent manner (70,78).

As mentioned, *P. aeruginosa* regulates the production of numerous virulence factors via the action of two separate, but coordinated QS systems, namely las and rhl. These systems control the transcription of genes in response to population density through the intercellular signals N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-(butanoyl)-Lhomoserine lactone (C4-HSL). The third *P. aeruginosa* signal, 2-heptyl-3-hydroxy-4-quinolone [*Pseudomonas* quinolone signal (PQS)], also plays a significant role in the transcription of multiple *P. aeruginosa* virulence genes. PQS is intertwined in the *P. aeruginosa* QS hierarchy with its production and bioactivity requiring the las and rhl QS systems respectively (79). PQS has also been demonstrated to induce the expression of rhlI, which encodes the C4-HSL synthase. These observations led to the conclusion that PQS acts as a connector signal between the las and rhl QS systems (80).

1.8.1. *Pseudomonas aeruginosa specific AHLs*

P. aeruginosa regulates the production of numerous virulence factors via the action of two separate, but coordinated QS systems, namely las and rhl. These systems control the transcription of genes in response to population density through the intercellular signals N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL-OdDHL), N-(butanoyl)-L-homoserinelactone (C4-HSL-BHL) and N-hexanoyl-L-Homoserine lactone (C6-HSL-HHL) (81). *Pseudomonas* quinolone signals (PQS) also play a significant role in the transcription of multiple *P. aeruginosa* virulence genes. PQS is intertwined in the *P. aeruginosa* Qs hierarchy with its production and bioactivity requiring the las and rhl QS systems respectively. The intercellular signals for the las and rhl quorum sensing systems are N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-(butanoyl)-L-homoserine lactone (C4-HSL) respectively (50,82). Together, these signals have been shown to control hundreds of genes representing 4 - 12 % of the *P. aeruginosa* genome (82). 3-oxo-C12-HSL(OdDHL) is involved not only in bacterial activation, but also in the subversion of the host immune system (82). Some infectious diseases have been shown to halt the onset of an autoimmune disease in animal. QS signal molecule OdDHL from *P. aeruginosa* inhibits T cell differentiation and can delay the onset of type 1 diabetes in the NOD mouse model (83). Analysis of the sequences of the lasR, lasI, rhlR and rhlI genes in *P. aeruginosa* has shown that when there is a defection in production of N-butanoyl-l-homoserine lactone (BHL) signaling molecule, there is also some defection in the production of virulence factors elastase, protease, pyocyanin and rhamnolipids. The combination of rhlR and rhlI mutations or only rhlI mutation probably explains their BHL and virulence factors deficiencies (84). *P. aeruginosa* also produces 2-heptyl-3-hydroxy-4-quinolone, a QS signal that regulates numerous virulence genes, including those involved in iron scavenging (85).

Bacterial processes related to motility, biofilm formation and cell aggregation will be affected or modified by the exogenous addition of AHLs (increasing the concentrations by using synthetic AHLs) (86).

1.9. Quorum quenching

Mammalian tissues and cells have developed multiple defense mechanisms as protection against bacterial infection. These include proteins and peptides such as lysozyme and defensins that kill bacteria (87). Recently, QS has been shown to be involved in the development of tolerance to various antimicrobial treatments and immunomodulation (8). Regulation of virulence via QS confers a strategic advantage over host defenses. Consequently, a drug capable of blocking QS is likely to increase susceptibility of infecting an organism to host defenses and its clearance from the host. The principles behind QS signal-mediated gene expression in both Gram-positive and Gram-negative bacteria are shared, but molecular mechanisms and signal molecules differ (88). Ambroxol, a mucolytic agent, has been reported to interfere with the formation of *P. aeruginosa*-derived biofilms in addition to reducing alginate production by undefined mechanisms (14). *P. aeruginosa* uses acyl-homoserine lactone-based QS systems to control its pathogenicity. One of them is N-3-oxo-dodecanoyl-homoserine lactone that can mediate bacterial QS, but it may also exert cytotoxic effects on mammalian cells. The monoclonal antibody RS2-1G9 generated against a 3-oxo-dodecanoyl-homoserine lactone analogue hapten is able to protect murine bone marrow-derived macrophages from cytotoxic effects and also prevent the activation of the mitogen-activated protein kinase p38. These data demonstrate the usefulness of immunopharmacotherapeutic approach to combat *P. aeruginosa* infections (89).

Inhibition of QS is also possible by synthesis of auto inducer analogs (AI-2) (90). Two of the most common quorum quenchers of AHL systems are AHL-lactonases and AHL-acylases (91). AHL-lactonases can hydrolyze the lactone ring while AHL-acylases hydrolyze the amide linkages. An AHL-lactonase was initially isolated from *Bacillus* (92) and has subsequently been found in several other bacterial species. AHL-acylases were initially discovered in *Variovorax paradoxus*,

which can utilize AHLs as a carbon source. *P. aeruginosa* have also been discovered to produce AHL-acylases (63).

Interference of QS signaling can also be achieved through the use of QS signal mimics (93). For example, the *P. aeruginosa las* QS molecule can inhibit the *Chromobacterium violaceum* AHL QS system (94). Boronic acids as potential AI-2 antagonists can stop QS in *V. harveyi* as the model organism, which produces bioluminescence upon autoinducer stimulation (37). The AI-2 molecule can exist in different forms in equilibrium and can complex with the boric acid. AI-2 binds with LuxP with the boron atom in its tetrahedral anionic form (95). Presumably, the stabilization of the negative charge is through interactions with the receptor binding site. Indeed, in the receptor-AI-2 complex, boron is next to two arginine residues (215 and 310) which can afford significant stabilization through ionic interactions. Boronic acids may serve as an excellent candidate for binding to LuxP because of the structural similarities between the boric acid complex and the boronic acid functional group (96).

2. MATERIAL AND METHODS

2.1. Hypotheses of this study:

2.1.1. *Pseudomonas aeruginosa expresses its virulence factors in dogs with otitis via quorum sensing and*

2.1.2. *Azithromycin attenuates the synthesis of AHLs in dogs with otitis and reduces the duration of treatment.*

The importance of the proposed research project rests on the fact that the investigation of herein proposed character would markedly complement the current knowledge with new information on pathophysiology of *Pseudomonas*-associated otitis and would propose novel treatment modalities for otitis in dogs.

2.2. Experimental design and methods

2.2.1. *Inclusion criteria*

a. Signed consent from patients' owners**.**
b. All Otitis cases associated with the *Pseudomonas aeruginosa* infection.

2.2.2. *Exclusion criteria*

a. Treatment with macrolide antibiotics 1 month prior to entering the study.
b. Previous treatment history not well documented.
c. Hypothyroidism or demodicosis as primary causes of otitis.
d. Concurrent diseases that would omit the use of planned medicaments (like Cushing’s disease).

e. Hypersensitivity to products used to treat otitis.

2.3. Sampling

a. After taking history and the initial physical examination, dogs were sedated (when necessary) to allow appropriate manipulation with the affected ear (history questions are attached in the appendix part).

b. Otoscopic examination was done by using the Heine otoscope. Clinical evaluation of the ear canal situation was described. All information was recorded on special data forms (forms are attached in the appendix part).

c. After the otoscopic evaluation of the ear canal, two sterile cotton swabs were inserted into the lumen and swabbed against the surface of the ear canal at the junction between the vertical and horizontal ear canals where the cartilage bends at about a 45º angle (21). First cotton swab was used for the culture and sensitivity testing and the second was rolled onto one glass slide that was later heat-fixed and stained with a modified Wright's stain (Diff-Quik^{R} ; Baxter Healthcare Co.).

d. Rinsing the ear canal with sterile saline and collection of flushing material.

30 ml of sterile normal saline was introduced to the ear canal by using sterile syringes. After collecting the liquids from the ear canal, sterile normal saline was added for making the final volume of 30 ml if the volume was lower than 30 ml.

e. Epidermal scrub

For the purpose of the parasitological examination, epidermal scrub was taken from the vertical ear canal using an ear curette. Then, dogs had their ears thoroughly rinsed with normal saline for the removal of the inflammatory debris. The ear canal was dried up

using suction via catheter. The epidermal scrub was examined microscopically under low magnification (x40) for the presence of parasites.

f. The last step of sampling

After the collection of samples, a thorough flushing followed to remove the inflammatory debris, the ear canal was dried up using a sterile non-traumatic catheter and suction was performed by a sterile syringe.

g. Blood collection

About 4 ml of blood was collected from the cephalic vein into EDTA and K_2EDTA vacutainers. Complete blood count (CBC) and biochemistry analyses ALT (Alanine aminotransferase), AP (Alkaline Phosphatase), urea and creatinine were performed. Blood was collected on the first, third and the last visit (days 0, 14 and 56). Blood parameters were determined using an autoanalyzer (ADVIA 120-SIEMENNS)

2.4. Treatment method and medications usage

2.4.1. *Methylprednisolone*

Methylprednisolone was prescribed in a dose of 1.0 mg/kg SID until the clinical observation rate reduced to less than 3 and then the dosage was reduced to 0.5 mg/kg and administered every 48 hours (97).

2.4.2. *Systemic antibiotic*

Based on culture results and sensitivity testing, treatment was started using a systemic antibiotic (ciprofloxacine/ enrofloxacine, 5 mg/kg SID), amoxicillin/clavulanate potassium 15 mg/kg, BID and ceftriaxone sodium 20 mg/kg BID (1)(97).

2.4.3. *Treatment with azithromycin*

All *Pseudomonas* positive otitis cases were treated randomly with instillation of 1 ml of azithromycin solution at the concentration of 50µg/ml or 1 ml of placebo (sterile saline) into the ear canal. A dose of 50µg/ml of azithromycin was selected for the preparation of antibiotic dilution due to the presence of debris in the ear (98).

2.4.4. *Client education*

To achieve optimum treatment, anatomy of the ear canal, pathophysiology of otitis and the manner of administering medications were explained to dogs' owners. Written instructions were handed to owners (Appendix 10.3).

2.5. Treatment and sampling timetable

Dogs were examined and samples collected weekly as described for the first (initial) work-up till the fourth week and then on weeks 6 and 8. Skin scrub for parasites was collected at the beginning of the study and on weeks 3 and 8 of the treatment.

2.6. Sample processing & laboratory protocols

2.6.1. *Cytological and parasitological examination*

Each cotton swab containing the ear sample was rolled onto the microscopic slide. The obtained sample was air dried, heat fixed and Diff-Quik stained for the cytology smear examination. The examination was performed in a semi-quantitative manner according to the published procedure (21,99) adjusted to an oil-immersion (x1000) magnification. Briefly, a microscopic slide was examined at a low (x100) magnification to locate most significant areas with the cell monolayer. Ten oil-immersion fields were examined and inflammatory cells, bacteria and yeasts were counted.

The mean number of cells and organisms per sample was calculated. Less than or equal 0.8 yeast / OIF (oil-immersion field) and less than 2 cocci / OIF were considered normal. More than 2 yeasts / OIF and more than 10 cocci / OIF as well as the presence of rods, even in small numbers, were considered pathologic since rods are not a part of the normal canine external ear canal flora (100). According to results found in literature, no inflammatory cells and no phagocytosis was expected to be seen in cured ears (99).

2.6.2. *Bacteriological examination*

Ear swabs were inoculated onto blood agar plates (Columbia agar supplemented with 5 % of sheep blood) and incubated aerobically at 37° C for 48+/-2 hours. After 24-h and 48-h incubation, plates were examined for the growth of *Pseudomonas spp*. or other pathogenic bacteria. Colonies morphologically consistent with *P. aeruginosa* were subcultured on fresh blood agar plates for subsequent identification.

Bacterial isolates were identified using the methods described by Quinn (101). Biochemical characteristics of the isolates were determined using the commercial Api 20NE kit (BioMerieux, France) according to the manufacturer's instructions.

2.6.3. *Anti-microbial susceptibility testing*

The cultured bacteria were tested for antimicrobial susceptibility using the disk-diffusion method by Kirby-Bauer. Briefly, a known concentration of antibiotic in a paper disk is applied on a bacteria lawn and after 24-hour incubation period, a zone of inhibition was measured and compared to the resistant/sensitivity index (102). The test was done in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI M100-S20). The following antimicrobial agents were tested, namely amikacin, gentamycine, tobramycin, enrofloxacin, ciprofloxacin, marbofloxacin, erythromycin, piperacilin, polymixin-B, trimetoprim-

sulfametoxasol, imipenem, ceftriaxone, cefotaxim, cefixin, cefibuten, ticarciline and azithromycin. (103).

2.7. Extraction of AHLs

2.7.1. *Ear sample processing*

Samples were collected from the ear by flushing with the sterile saline solution (30 ml), centrifuged at 3100×g for 30 min at 4˚ C (Sigma, 2K15G), and the sample supernatant was stored at -80˚ C till extraction. The extraction was performed by dichlormethane and the solvent was removed by evaporation under vacuum according to Erickson (104). The obtained extracts were reconstituted in 50 µl of organic solvent and stored at -20˚ C till further analysis.

2.7.1.1. Apparatus

Syncore Reactor R-48 (Büchi, Flawil, Switzerland) was used for the evaporation of extracts. Measurements were performed with the liquid chromatography system Acquity (Waters, Milford, MA, USA) equipped with an analytical column ZORBAX Eclipse C18, Rapid Resolution HD, 1.8 µm, 2.1 x 100 mm (Agilent) and mass selective detector Xevo TQ MS (105). The programs MassLynx and TargetLynx were used for the system control and for data processing respectively. The mobile phase flow rate was 0.25 mL/min, the injection volume 10 µL and the column temperature 30° C. The elution mode started with isocratic profile of methanol-water (35+65) for 4 min, followed by a linear gradient from 35 % to 95 % methanol over 4 min and back to 35 % over 1 min. Isocratic profile of methanol-water (35+65) was used again for 2 min. Mass-spectrometric detection was done in the positive ion mode. The capillary voltage was 0.4 kV and the ions monitored are given in the table below.

Table 1: Ions monitored for the selected AHLs

Substance	Retention time (min)	Precursor ion mass	Product ion mass	Cone voltage (V)	Collision cell voltage (V)
C4-HSL(BHL)	1.98	172.096	101.980	18	**10**
		172.096	144.073	18	**8**
C6-HSL(HHL)	4.75	200.160	98.986	18	**6**
		200.160	101.984	18	**10**
3-oxo-C12-HSL	9.16	298.266	102.038	26	**12**
		298.266	**197.138**	**26**	**14**

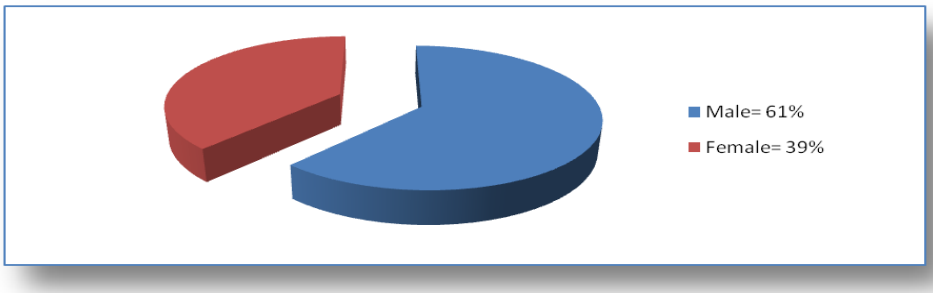
2.7.1.2. Reagents

The standard compounds C4-HSL, C6-HSL, and 3-oxo-C12-HSL were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions of individual AHLs (concentration 1 mg/mL) were prepared in methanol and mixed, and working standard solutions (concentrations from 1 to 60 ng/mL) were prepared in the mixture of methanol and deionised water (35+65).

2.7.1.3. Case distribution

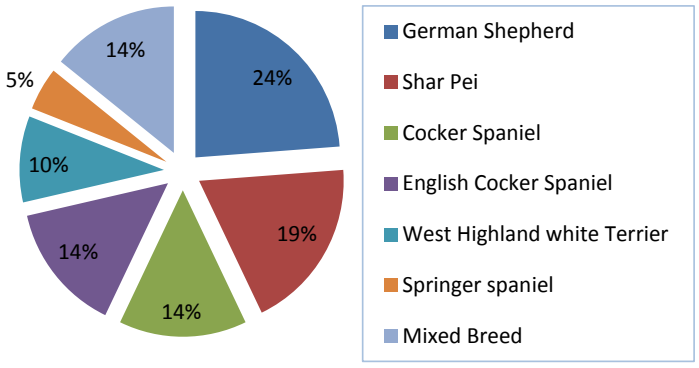
Twenty-one dogs (mean age: 8.85 years, mean weight 20.23 kg) were included in this study.

Figure 2: Case Distribution by Gender



Breeds included German shepherd (5/21), Shar Pei (4/21), Cocker Spaniel (3/21), Highland Terrier (2/21), English Cocker Spaniel (3/21), Springer spaniel (1/21) and mixed-breed (3/21). Twelve dogs (12/21) were treated orally with antibiotics. Also twelve dogs (12/21) were treated with azithromycin and nine dogs (9/21) were treated with placebo. Fourteen dogs (14/21) were treated with methylprednisolone: six (6/9) from the group were not treated with azithromycin and eight (8/12) from the group were treated with azithromycin. Fifteen dogs (15/21) were treated with fluoroquinolone antibiotics (enrofloxacin or ciprofloxacin orally): five (5/9) from the group were not treated with azithromycin and ten (10/12) from the group were treated with azithromycin. Atopic syndrome was determined to be the primary cause for the disease in 12 dogs (11/21). The primary cause for the disease could not have been determined in ten dogs (10/21).

Figure 3: Case Distribution by Breed



2.8. Animal use

This project was evaluated by the Ljubljana Veterinary Faculty's ethical Committee and gained the approval No. (34401-19/2010/2). Signed consents from dogs' owners were required.

2.9. Statistical design

The study was stated as a randomized, double blind, placebo-controlled and prospective clinical study. A hundred and thirty-five samples (ear lavage) from 21 dogs with *Pseudomonas aeruginosa*-associated otitis were collected from winter 2010 till spring 2012.

Dogs were divided into two groups:

G1: Topical treatment with placebo (N=9)

G2: Topical treatment with azithromycin (N=12)

The effect of treatment with azithromycin (treatment) and time on the continuous outcomes of observed variables was verified with the linear mixed effects model, where dogs/patients were considered random effect components. Interaction between the treatment and the time was also considered in the model. The variables, methylprednisolone and antibiotic treatment, were included in the model as a controlling covariate in order to remove the possible confounding effect of those variables. The pre-planed between-times and between-treatment comparisons were carried out with the contrast analysis. The effect of treatment and time on binary outcomes was estimated with the logistic regression with the random effect. Similarly to the case of the continuous outcomes, the variables, methylprednisolone and fluoroquinolone antibiotics treatment, were included as a controlling covariate to remove the confounding effect. The pre-planed between-time and between-treatment differences were estimated with the contrast analysis. BHL, HHL and OdDHL were also analyzed as covariates to assess their influence on other selected variables. An analysis was performed only for numerical outcomes with enough variability (rod-shaped bacteria, cocci, overall clinical score, neutrophil granulocytes).

The effect of time and treatment with azithromycin on binary outcomes was estimated with the logistic regression with the random effect. Similarly to the case of the continuous outcomes, the variables, methylprednisolone and systemic antibiotic treatment, were included as a controlling covariate to remove the confounding effect. The pre-planned between-time and between-treatment differences were estimated with the contrast analysis. The P-value equal or less than 0.05 was considered as statistically significant. All analyses were performed with R language for statistical computing (106).

3. RESULTS

Sampling and clinical treatment lasted from October 2010 till April 2012. A hundred and thirty-five ear flushings for the detection of AHLs, 131 ear swabs for cytology, 59 blood samples and 40 parasitological skin scrubs were collected from 21 dogs and checked based on the research protocol.

3.1. Case information

3.2. Table 2: Case information

Set Number	BREED	AGE (year)	SEX	WEIGHT/kg	Infected ear	No. of ear	No. of
1/37	German Shepherd	5	M	33	LEFT	5	3
2/12	Cocker Spaniel	14	M	14	RIGHT	5	2
3/19	Cocker Spaniel	7	F	16.4	LEFT	7	3
4/20	Cocker Spaniel	7	M	15	LEFT	7	3
5/13	Highland Terrier	10	M	7.36	LEFT	7	3
6/50	Highland Terrier	10	M	7.36	RIGHT	7	2
8/37	Mix	3	F	13.7	LEFT	7	3
11/47	Shar Pei	8	M	12	LEFT	6	3
12/122	German Sheperd	7	F	42	LEFT	7	3
15/26	German Sheperd	6	M	48	LEFT	6	3
16/46	Shar Pei	8	M	12	LEFF	6	3
18/14	Mix	11	M	16.5	LEFT	7	3
19/4	Mix	11	F	16.5	RIGHT	7	3
20/14	German Sheperd	10	M	41	LEFT	7	3
21/42	German Sheperd	10	M	41	RIGHT	7	3
22/48	Shar Pei	5	F	21	LEFT	7	3
23/17	Shar Pei	5	F	21	RIGHT	7	3
26/1	English Cocker Spaniel	13	M	14.2	LEFT	5	2
28/38	Springer Spaniel	9	M	23	RIGHT	7	3
29/5	English Cocker Spaniel	14	F	10	RIGHT	7	3

3.3. Antibiotic usage

3.4. Table 3: Antibiotic usage

Set number	Name of antibiotic	Duration of usage
1/37	CIPROFLOXACIN	4WEEKS
2/12	CEFTRIAXONE	4WEEKS
3/19	NOT USED	NOT USED
4/20	ENROFLOXACIN	2WEEKS
5/13	ENROFLOXACIN	2WEEKS
6/50	ENROFLOXACIN	2WEEKS
8/37	NOT USED	NOT USED
11/47	NOT USED	NOT USED
12/122	CIPROFLOXACIN	2WEEKS
15/26	NOT USED	NOT USED
16/46	NOT USED	NOT USED
18/14	ENROFLOXACIN	4WEEKS
19/4	ENROFLOXACIN	4WEEKS
20/14	ENROFLOXACIN	4WEEKS
21/42	ENROFLOXACIN	4WEEKS
22/48	CIPROFLOXACIN	3WEEKS
23/17	CIPROFLOXACIN	3WEEKS
26/1	NOT USED	NOT USED
28/38	CIPROFLOXACIN	3WEEKS
29/5	SYNULOX	4WEEKS

3.5. Clinical observation

3.5.1. *Otoscopic examination*

Table 4: Clinical observation based on otoscopic examination

Case Number	**First visit Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Last visit Week 8**
1/37	E2,O1,p1,EX1	E2,O1,p1,E1	E1,O1,P1,	E1,O1,P1,	E1,P1,	End of study	End of study
2/12	E2,O1,p1,S2	NA	S2,E2,O1,P1,EX	E1,	S1,E1,	End of study	End of study
3/19	S2,E1,01,p2,ex1	S2,E2,O1,P1,E	S2,E1,O1,P2,EX	S1,E1,O1,EX1,	E1,O1,	Normal	Normal
4/20	S3,E3,o2,p3,ex2	S3,E2,O2,P2,E	E1, P1,EX1,	S1,E1,P1,	Normal	E1,	E1,
5/13	S4,E3,O4,P4,	S2,E1,O1,P1,E	S2,E2,O3,P2,EX	S1,E2,O1,P1,	E1,	S1,	S1,e1,o1,
6/50	S4,E4,p4,ex3,	S2,E1,O1,P1,E	S2,E2,O2,P1,	S2,E2,O2,P1,	S2,E2,O2,	E1,O1,	S1,e1,01,
8/37	E1,EX1,	E1,	E1,	E1,	Normal	E1,	E<1,
11/47	S1,e2,01,	S2,E2,O1,P1,	S2,E2,O2,P1,	E1,O1,EX1,	S1,E1,O1,	S1,E1,O1,EX1,	Normal
12/122	S3,E3,o2,p2,ex2,	S1,E1,O1,	E1,	E1,P1,	Normal	S1,E1,	Normal
15/26	S2,E2,o2,p1,ex1,	E2,O1,	S1,E1,P1,	S1,E1,	NA	S1,E2,O1,	E1
16/46	S2,E2,o1,	S1,E2,O1	NA	E1,	S1,E1,	O1,p1,	Normal
18/14	E1,	E1,P1,	E1,	S1,E1,EX1,	EX1,	Normal	Normal
19/4	E1,	E1,	E1,EX1,	E1,EX1,	E1,	Normal	Normal
20/14	S2,E2,o1,ex2,	E1,O1,EX1,	E1,O1,EX1,	E1,	Normal	E1,	E1,ex1,
21/42	S1,E2,01,ex1,	E1,	E1,	EX1,	Normal	EX1,	Normal
22/48	S2,E2,ex2,	S2,E2,O1,	S1,E2,O1,	S1,E1,	E1,	Normal	Normal
23/17	S2,E2,o2,ex2,	S2,E2,O2,EX1	S1,E1,O1,	E1,O1,	Normal	E1,Ex1,	Normal
26/1	E2,	E1,O2,	E1,O1,	E1,	E1,	Normal	Normal
28/38	S3,E3,O3,P2,	S2,E2,O2,P1,	S1,E2,O2,P1,	S3,E3,O2,ex1,	S2,E2,	S1,E1,	S1,E2,EX1
29/5	S1,E2,O1,P2,Ex1,	E1,P1,EX1,	Ex1,	Normal	Normal	E1,	E2,P1,

Legend				
	N	Normal	O	Oedema
	NA	Not available	P	Pain
	E	Erythema	EX	Exudate
	S	Stenosis	D	Day
	Y	Yes=used	W	week
	Clinical	1=mild, 2=moderate, 3=severe, 4=very severe		

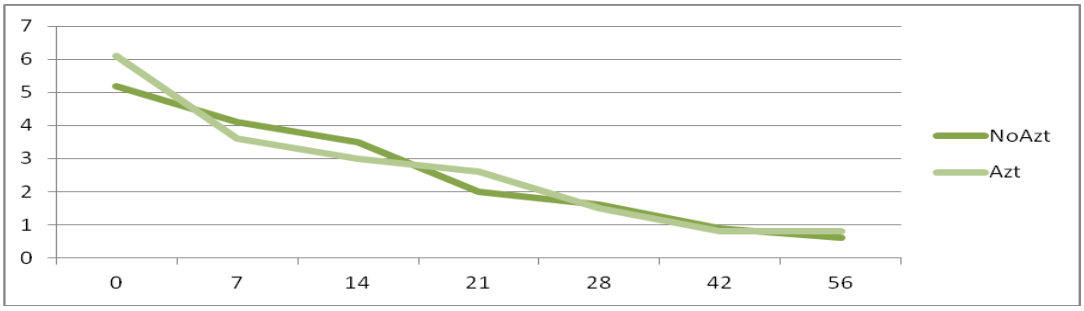
3.5.2. *Effect of treatment on clinical signs*

Treatment with azithromycin had no effect on the ear canal erythema (P=0.8), oedema (P=0.4), stenosis (P=0.8) or pain (P=0.2). Time had a significant effect on all clinical signs individually (P≤0.005). Treatment with methylprednisolone had no effect on erythema (P=0.2), oedema (P=0.3) and stenosis (P=0.2); however, it had a significant effect on pain (P=0.02). Similarly, treatment with fluoroquinolone antibiotics had no effect on erythema (P=0.9), oedema (P=0.9) and stenosis (P=0.8); however, it had a significant effect on pain (P=0.4) (Table 5).

Clinical signs estimated together as one overall variable were also not affected by treatment with azithromycin (P=0.6) nor were they affected by the treatment with methylprednisolone (P=0.1) or fluoroquinolone antibiotics (P=0.9). The severity of overall clinical signs diminished over time (P<0.0001) (Table 5).

3.3.3. Clinical signs evaluation by graph

Figure 4: Clinical score



(vertical, average score-horizontal, time), overall clinical score combining erythema, oedema, pain and stenosis. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12), data expressed as ± SEM.

Table 5: Clinical signs (Score 0-4)

Clinical Signs(Score 0-4)							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
Erythema*							
NoAzt	2.0±0.2	1.6±0.2	1.3±0.2	1.0±0.2	0.8±0.2	0.5±0.2	**0.3±0.2**
Azt	2.2±0.2	1.5±0.2	1.4±0.1	1.2±0.2	0.7±0.2	0.4±0.1	**0.5±0.2**
Oedema*							
NoAzt	1.1±0.4	1.0±0.2	0.9±0.3	0.6±0.2	0.2±0.1	0.1±0.1	**0.1±0.1**
Azt	1.2±0.3	0.8±0.2	0.5±0.2	0.5±0.2	0.2±0.2	0.2±0.1	**0.2±0.1**
Pain* [@]							
NoAzt	1.0±0.5	0.6±0.2	0.6±0.3	0.2±0.1	0.4±0.3	0.0±0.0	**0.1±0.1**
Azt	1.1±0.4	0.4±0.2	0.5±0.2	0.3±0.1	0.0±0.0	0.1±0.1	**0.0±0.0**
Stenosis*							
NoAzt	1.1±0.5	1.0±0.3	0.8±0.3	0.2±0.1	0.1±0.1	0.3±0.2	**0.1±0.1**
Azt	1.7±0.4	1.0±0.3	0.6±0.2	0.7±0.3	0.5±0.2	0.2±0.1	**0.2±0.1**
ClinScore*							
NoAzt	5.2±1.4	4.1±0.6	3.5±0.9	2.0±0.5	1.6±0.6	0.9±0.3	**0.6±0.4**
Azt	**6.1±1.1**	**3.6±0.8**	**3.0±0.7**	**2.6±0.7**	**1.5±0.6**	**0.8±0.3**	**0.8±0.3**

Legend table 5: data expressed as mean ± SE. Ear and ear canal clinical signs score (0 (the variable (clinical sign) absent) – 4 (the variable (clinical sign) very evident). ClinScore: overall clinical score combining erythema, oedema, pain and stenosis. NoAzt: not treated with antibiotics (n=9). Azt: treated with antibiotics (n=12). *Significant effect of time on variable. [@]significant effect of methylprednisolone on variable.

3.6. Bacteriological results

3.6.1. *Culture and isolation of microorganisms*

Table 6: Culture and isolation

Set Number	Strains of bacteria													
	Week 0		**Week 1**		**Week 2**		**Week 3**		**Week 4**		**Week 6**		**Week 8**	
	PS.	**OB.**	**PS.**	**OB.**	**PS.**	**OB.**	**PS.**	**OB.**	**PS.**	**OB.**	**PS.**	**OB.**	**PS.**	**OB.**
1/37	+	-	+	-	+	-	+	-	+	-	-	-	-	-
2/12	+	**SP.**	+	-	-	-	-	**M.**	-	**M.**	-	-	-	-
3/19	+	-	+	-	+	-	+	-	+	-	+	-	-	-
4/20	+	-	+	-	+	-	+	-	+	-	+2	-	+2	-
5/13	+	-	+	**P.**	+2	-	+2	**P.**	+2	**P.**	+2	-	+	-
6/50	+	-	+2	-	+	**P.**	+	**P.**	+2	**P.**	+2	-	+2	-
8/37	+2	-	+2	-	+2	-	+	-	+	-	+	-	+	-
11/47	+	**Sp,cr.**	+	-	**NA**	**NA**	+	-	+	**M.**	-	-	-	-
12/122	-	**sp**	+	**sp**	+	-	+	-	-	-	+	**E.**	+	-
15/26	+	**E.**	+2	-	+2	-	-	-	**NA**	**NA**	+	-	-	-
16/46	+	**P.**	-	**P.**	+	-	**NA**	**NA**	-	-	+	**P.**	-	-
18/14	+	-	+	-	+	-	+	**P.**	+	-	-	**P.,SP.**	-	-
19/4	+	**S.**	+	-	+	**P.**	+	-	-	-	-	-	-	**P.**
20/14	+	-	+	**Cr.**	-	-	+	-	-	**Sp.**	+2	-	-	-
21/42	+	-	+	**P.**	-	-	+	-	+	-	+2	-	-	-
22/48	+	-	-	-	+	**P.**	_	**P.**	-	**P.s.cr.**	-	-	-	-
23/17	+	-	+	-	+	**P.**	-	**P.**	-	**p.**	-	**P.**	-	**P.**
26/1	+	-	+	**sp.**	+	-	-	-	-	-	-	-	-	-
28/38	+	-	+	**P.**	+	**P.**	+	**P.**	+	**P.**	+	**Sp.**	+	**P.**
29/5	+	**P.**	+	**P.**	+	**P.**	+	**P.**	+	**P.**	+	**P.,Sp.**	-	**P.**

Legend				
	Ps	*Pseudomonas aeruginosa*	OB.	*Other Bacteria*
	Sp	*Staphylococcus pseudintermedius*	P	*Proteus*
	M	*Malassezia pachydermatis*	Cr	*Corynebacterium spp.*
	Pro.	*Providencia*	NA	*Not available data*
	E	*Escherichia coli*	S	*Streptococcus canis*
	+2	*Two different type of bacteria*	+,-	*Presence, absence*

Comments on table 6: Due to a technical problem in the laboratory, the first culture of the case 12/22 was negative. Supported by positive cytology results, the case was included in the study and confirmed to be *Pseudomonas aeruginosa* positive by subsequent cultures.

3.6.2. *Antibiotic susceptibility results*

Table 7: Antibiotic susceptibility results

Antibiotic / Set No:	Cip.	S	Cef.	S	Enr.	S	Gen.	S	Az.	S	Tob.	S	Mar.	S
1/37	16	R	21	S	16	R	8	R	6	R	18	S	20	S
1/37	19	I	20	I	19	R	15	S	6	R	18	S	10	R
2/12	27	S	15	I	12	R	24	S	6	R	28	S	16	I
2/12	29	S	17	I	13	R	25	S	6	R	27	S	16	I
3/19	20	I	6	R	6	R	26	S	6	R	31	S	6	R
3/19	20	S	18	I	6	R	20	S	6	R	24	S	6	R
4/20	34	S	21	S	21	S	22	S	6	R	26	S	27	S
4/20	12	R	8	R	18	I	6	R	6	R	6	R	13	R
4/20	29	S	19	I	18	I	18	S	6	R	23	S	20	S
5/13	32	S	16	I	24	S	23	S	6	R	26	S	27	S
5/13	22	S	20	I	no	-	21	S	6	R	no	-	14	R
5/13	24	S	21	S	15	R	22	S	6	R	28	S	17	I
6/50	32	S	16	I	24	S	23	S	6	R	26	S	27	S
6/50	28	S	18	I	no	-	19	S	6	R	24	S	19	S
6/50	27	S	16	I	no	-	19	S	6	R	23	S	17	I
8/37	24	S	14	I	10	R	19	S	6	R	24	S	9	R
8/37	20	S	11	R	10	R	21	S	6	R	25	S	11	R
8/37	24	S	15	I	10	R	20	S	6	R	26	S	16	I
11/47	40	S	20	I	24	S	17	S	6	R	22	S	26	S
11/47	35	S	23	S	24	S	25	S	-	-	20	S	23	S
12/12	31	S	22	S	21	S	17	S	6	R	23	S	25	S
12/12	27	S	23	S	17	I	18	S	-	-	22	S	22	S
12/12	39	S	26	S	28	S	26	S	6	R	28	S	30	S
15/26	36	S	25	S	27	S	24	S	6	R	27	S	28	S
15/26	19	I	18	I	6	R	19	S	-	-	15	S	25	S
16/46	40	S	20	I	24	S	17	S	6	R	22	S	26	S
16/46	35	S	23	S	24	S	25	S	-	-	20	S	23	S
18/14	33	S	19	I	22	S	23	S	-	-	21	S	28	S
18/14	30	S	18	I	20	S	20	S	-	-	18	S	20	S
19/4	33	S	19	I	22	S	23	S	-	-	21	S	23	S
19/4	20	I	20	I	17	I	20	S	-	-	20	S	16	S
20/14	21	S	18	I	11	R	19	S	6	R	19	S	20	S
20/14	34	S	26	S	-	-	23	S	6	R	22	S	27	S
21/42	21	S	18	I	11	R	19	S	-	-	19	S	20	S
21/42	34	S	26	S	-	-	23	S	-	-	22	S	27	S

22/48	25	S	22	S	11	R	21	S	6	R	26	S	17	I
23/17	30	s	6	R	20	R	20	s	6	R	24	s	23	s
26/1	35	S	24	I	26	S	29	S	-	-	23	S	23	S
26/1	31	S	24	S	14	S	27	S	-	-	20	S	18	S
28/38	29	S	16	I	18	I	18	S	-	-	18	S	19	S
29/5	32	S	18	I	24	S	25	S	-	-	19	S	21	S

In each case, a susceptibility test was run at least on the first and the last visit. All tested antibiotics and the results are shown in the appendix (Table: 7). This table shows information on antibiotics selected for treatment in our study (41 susceptibility tests).

3.6.3. *Bacterial culture and sensitivity*

All dogs had *Pseudomonas*-associated otitis externa at the beginning of the study. *P. aeruginosa* was isolated from ear canals of ten (10/21) dogs on day 56: six (6/9) were from the group not treated with azithromycin and four (4/12) from the group treated with azithromycin. Treatment with azithromycin did not influence the presence of *P. aeruginosa* in the ear canal on day 56 of the study (OR (odds ratio) = 0.1; 95% CI: 0.02-1.2; P=0.07) nor was the likelihood of *P. aeruginosa* presence in the ear canal on day 56 of the study influenced by the treatment with methylprednisolone (OR=3.0; 95% CI: 0.3-26; P=0.3) or fluoroquinolone antibiotics (OR=1.4; 95% CI: 0.2-12; P=0.7)

The presence of rod-shaped bacteria other than *P. aeruginosa* in the ear canal was also assessed. Nine (9/21) dogs had rod-shaped bacteria present in their ear canals at the conclusion of the study (day 56). Three dogs (3/9, 3x *Proteus sp*.) had positive culture in the group not treated with azithromycin and six dogs (6/12, 3x *Proteus sp*., 2x *E. coli*, 1x *Corynebacterium)* had positive culture in the group that was treated with azithromycin. Treatment with fluoroquinolone antibiotics did not influence the presence of other rod-shaped bacteria on day 56 of the study (OR=4.7; 95% CI: 0.68-4.0; P=0.1) nor was the likelihood of the presence of other rods at the end of the study influenced by the treatment with methylprednisolone (OR=0.78; 95% CI: -2.1-1.7; P=0.9).

Seven dogs (7/21) had been recognized with the resistancy to more than one antibiotic *in vitro*. *P. aeruginosa* present in their ear canal on the initial bacteriology: three (3/9) were from the group not treated with azithromycin and four (4/12) from the group treated with azithromycin.

Eight dogs (8/21) had resistant *P. aeruginosa* present in their ear canal on the bacteriology of the day 56 sample: three (4/9) were from the group not treated with azithromycin and four (4/12) from the group treated with azithromycin. Treatment with azithromycin was not shown to increase the likelihood for *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones (OR=1.4; 95% CI: 0.1-12; P=0.9). Treatment with fluoroquinolone antibiotics was not shown to increase the likelihood for *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones on day 56 of the study (OR=0.3; 95% CI: 0.02-4.4; P=0.4) nor was the likelihood for *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones on day 56 after the treatment with methylprednisolone (OR=8.2; 95% CI: 0.5-142; P=0.2)

3.6.4. *Parasitology evaluation*

No parasites were detected from ear canal epidermal scrubs from any of the patients at any time of the study.

3.7. Cytology investigation (slides of samples)

3.8. Cytology investigation

3.8.1. *Cytology of microorganisms*

Table 8: **Cytology of microorganisms**

Set	**Type of**	First visit						Last visit
1/37	**Rods**	6.8	16.4	8.9	4.4	1.9	0	0
	Cocci	1	8.1	123.1	4.4	1.9	0	0
	Yeast	0	0	0	0	0	0	0
2/12	**Rods**	40	28.5	5	0.6	0.5	0	0
	Cocci	55.5	24	9.8	3	4.3	0	0
	Yeast	0	0	0	0	0.7	0	0
3/19	**Rods**	18.8	20.9	25.1	2.5	1.6	15.7	0.3
	Cocci	0	0	0	0	0	0.3	3.1
	Yeast	0	0	0	0	0	0	0.4
4/20	**Rods**	41.5	3.1	3.4	12.2	3.7	6.7	2.2
	Cocci	11.7	5.2	23.7	22.8	5.5	2.6	0
	Yeast	0.2	0	0.2	0	0	0	0
5/13	**Rods**	468	34.4	38	7.8	1	15.5	14.3
	Cocci	0	0	0	0	0	0	0
	Yeast	0	0	0	0	0	0.1	0
6/50	**Rods**	124	8.7	11.9	9.7	2.7	5.3	3.9
	Cocci	0	0	0	0	0	0	0
	Yeast	0	0	0	0	0	0	0
8/37	**Rods**	17.4	2.4	0.7	1.5	1.9	9.2	2.8
	Cocci	0	0	0	0	0	0	0
	Yeast	0	0	0	0	0	0	0
11/47	**Rods**	72	32.9	0	54.1	47.4	19.2	4.9
	Cocci	16.1	0	0	0	0	0	0
	Yeast	0	0	0	0	0	0	0
12/122	**Rods**	206	29.3	10.2	12.6	5.4	31.3	5.7
	Cocci	183	10.1	1.2	7.4	0	3.5	8.1
	Yeast	0	0	0	0	0.1	0	0
15/26	**Rods**	53.7	45.2	50.1	0	nd	92.5	79
	Cocci	0.7	0	0	0	nd	0	0
	Yeast	0.4	0.3	0	0.1	nd	0	0
16/46	**Rods**	53.7	15.2	NA	32.8	12.9	32.3	72.1

	Cocci	12.8	10.5	NA	0	0	0	3.4
	Yeast	13.3	1.8	NA	0	0	0	0
18/14	**Rods**	11.3	9.7	7.2	3.3	0.3	2.8	0.3
	Cocci	0	3.8	0	7	5.7	5.5	0
	Yeast	1.7	0.4	1.1	6.3	0	0	0
19/4	**Rods**	7.3	4.9	NA	0.2	0	0	0
	Cocci	3.6	5.2	NA	4.2	3.7	7.3	2.2
	Yeast	0	0	NA	0	0	0	0
20/14	**Rods**	39.9	170.6	78.7	23.6	7.4	8.3	0
	Cocci	10.2	6.9	40.5	27.7	20.2	3.7	4.4
	Yeast	0	1.2	0	0	0.2	0.4	0
21/42	**Rods**	147.3	202.5	28	12.4	15	22	0
	Cocci	23.3	0	20.6	18.2	27.4	8.5	4.9
	Yeast	0	0	0	0	0	0	0
22/48	**Rods**	445	58.1	53.5	66.8	31.1	55.1	4.3
	Cocci	46	22.7	0	25.3	14	0	0
	Yeast	0	0	0	0	0	0	0
23/17	**Rods**	109.5	14.4	53.2	37.1	28.3	20.7	3.5
	Cocci	0	0	0	0	0	8.7	0
	Yeast	0	0	0	0	0	0	0
26/1	**Rods**	14.4	14.4	1.7	0.8	0	0	0
	Cocci	0	0	0	0	0	0	0
	Yeast	0	0	0	0	0	0	0
28/38	Rods	4.3	76	107	89.2	32.2	136	88.1
	Cocci	0	0	0	0	0	12.5	5.4
	Yeast	2.7	0.9	0	0	0	0.1	0
29/5	**Rods**	364	75.5	17.7	23.8	20.7	92	43.7
	Cocci	0	0	0	15.1	5.5	39.5	0
	Yeast	0	0	0	0	0	0	0

All dogs (21/21) had rod-shaped bacteria in their ear canal. Treatment with azithromycin had no effect (P=0.9), whereas time had a significant effect on the reduction of rod-shaped bacteria in the ear canal (P<0.0001) (Table 9). Fourteen (14/21) dogs had rod-shaped bacteria in their ear canals on day 56; out of those, four (4/12) were and three (3/9) were not treated with azithromycin. Treatment with methylprednisolone (P=0.9) or fluoroquinolone antibiotics (P=0.2) had no effect on rod-shaped bacteria in the ear canal.

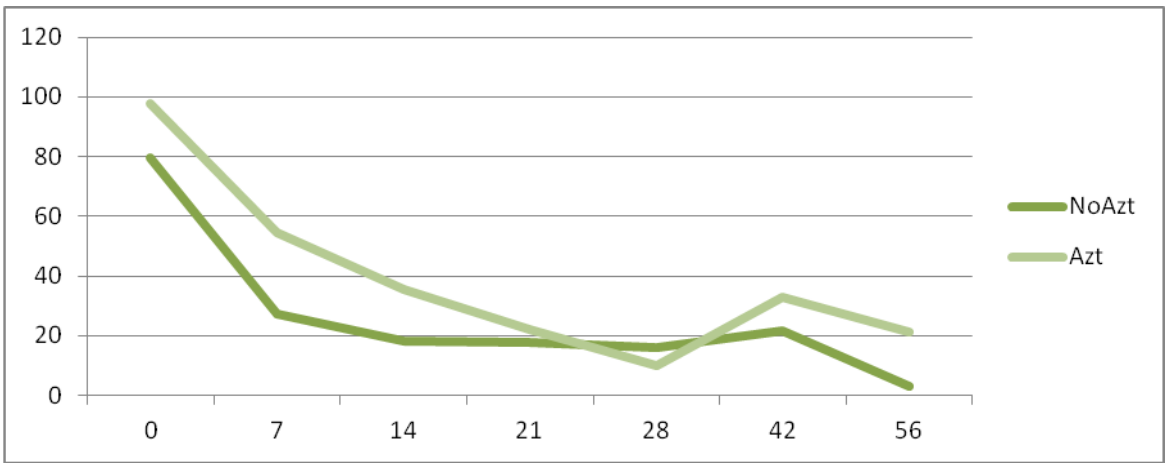
Table 9 : Ear cytology (No./OIF) - Rods*

Ear cytology (No./OIF) - Rods*							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
NoAzt	79.7	27.1	18.3	17.8	15.8	21.5	3.2
Azt	97.8	54.3	35.5	22.0	10.1	32.7	21.3

Legend table 9: data expressed as mean ± SE. Rods: rod-shaped bacteria. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). * Significant effect of time. No./OIF: No. (number) / OIF (Oil Immersion Field) - 1000x magnification.

3.8.2. *Ear cytology evaluation by graph (Rods)*

Figure 5: Rod-shaped bacteria



NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). OIF (Oil Immersion Field) - 1000x magnification. (vertical, average rods, NO. /OIF-horizontal, time). Data expressed as ± SEM.

Twelve dogs (12/21) had cocci present in their ear canal at the beginning of the study: three (3/9) from the group not treated with azithromycin and nine (9/12) from the group treated with azithromycin. Treatment with azithromycin (P=0.07) and time (P=0.1) had no effect on cocci in the ear canal (Table 10). Seven (7/21) dogs had cocci in their ear canals on day 56; out of those, six (6/12) were treated and one (1/9) was not treated with azithromycin. Treatment with methylprednisolone (P=0.9) had no effect on cocci, whereas treatment with fluoroquinolone antibiotics affected cocci in the ear canal (P=0.02).

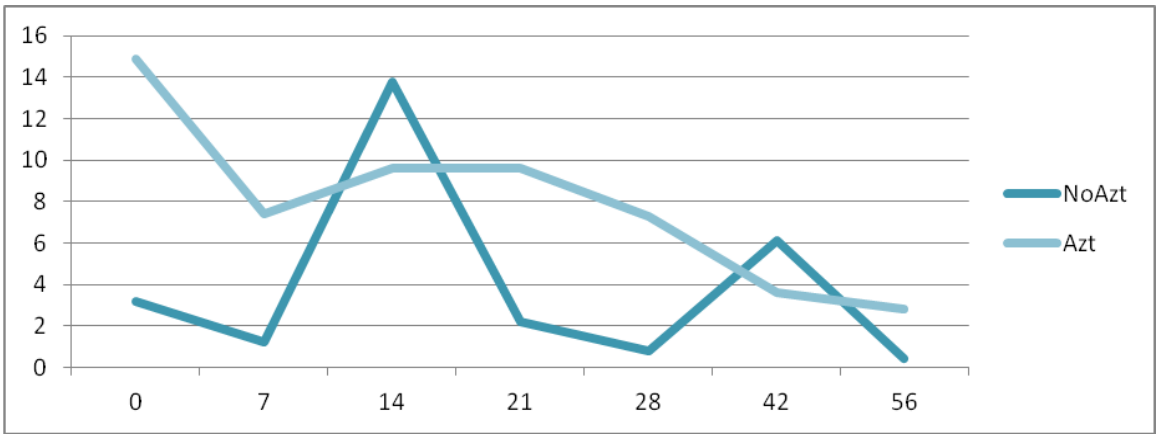
Table 10: Ear cytology (No. /OIF) - Cocci[A]

Ear cytology (No./OIF) - Cocci[A]							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
NoAzt	3.2	1.2	13.8	2.2	0.8	6.1	0.4
Azt	14.9	7.4	9.6	9.6	7.3	3.6	2.8

Legend table 10: data expressed as mean ± SE. Cocci: spherically shaped bacteria. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). A significant effect of treatment with fluoroquinolone antibiotics. No./OIF: No. (number) / OIF (Oil Immersion Field) - 1000x magnification.

3.8.3. *Ear cytology evaluation by graph (Cocci)*

Figure 6: Spherically shaped bacteria



NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). A significant effect of treatment with fluoroquinolone antibiotics. OIF (Oil Immersion Field) - 1000x magnification. (vertical, average cocci, NO./OIF-horizontal, time). Data expressed as ± SEM.

Four dogs (4/21) had yeasts present in their ear canal: zero (0/9) from the group not treated with azithromycin and four (4/12) from the group treated with azithromycin. The presence of yeasts was variable and occasionally rendered positive results on different dogs at different time intervals. Neither treatment with azithromycin (P=0.2) nor time (P=0.5) had an effect on the presence of yeasts in the ear canal (Table 11). One dog (1/21) had yeasts in the ear canal on day 56; the dog was not treated with azithromycin. Treatment with methylprednisolone (P=0.9) or fluoroquinolone antibiotics (P=0.2) had no effect on the presence of yeasts in the ear canal.

Table 11: Ear cytology (No./OIF) - Yeasts

Ear cytology (No./OIF) - Yeasts							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
NoAzt	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Azt	1.3	0.4	0.1	0.5	0.1	0.0	0.0

Legend table 11: data expressed as mean ± SE. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). No./OIF: No. (number) / OIF (Oil Immersion Field) - 1000x magnification.

3.8.4. *Cytology of defense cells*

Table 12: Cytology of defense cells

Set number	Type of defense cell	**First visit Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Last visit Week 8**
1/37	**Neutrophil**	0.6	1.5	3.7	1.4	0.7	0	0
	Macrophage	0	0	0	0	0	0	0
2/12	**Neutrophil**	0.7	0.5	0.2	0	0.1	0	0
	Macrophage	0	0	0	0	0	0	0
3/19	**Neutrophil**	4.7	0.4	0.9	0.3	0.4	0.7	0.1
	Macrophage	0	0	0	0	0	0	0
4/20	**Neutrophil**	0.9	0.7	0.8	0.5	0.6	0.9	0.5
	Macrophage	0	0.1	0.1	0	0	0	0
5/13	**Neutrophil**	0.5	0.4	0.6	0.3	0.1	0.3	0.7
	Macrophage	0.1	0	0	0	0	0	0
6/50	**Neutrophil**	1	0.8	0.9	0.6	0.3	0.6	0.2
	Macrophage	0	0.1	0.1	0	0	0	0
8/37	**Neutrophil**	0.6	1	0.4	0.3	0.3	0.6	0.4
	Macrophage	0	0	0	0	0	0	0.1
11/47	**Neutrophil**	1	0.5	0	1.3	0.7	0.5	0.3
	Macrophage	0	0	0	0	0	0	0
12/122	**Neutrophil**	1.8	0.9	0.9	0.7	0.4	1.4	0.5
	Macrophage	0	0	0	0	0	0	0
15/26	**Neutrophil**	0.6	0.4	0.2	0.1	NA	0.8	2.5
	Macrophage	0	0	0.1	0	NA	0	0
16/46	**Neutrophil**	1.2	1.1	NA	1.3	0.8	0.9	1.3
	Macrophage	0	0	0	0.1	0	0	0
18/14	**Neutrophil**	0.5	0.3	0.4	0.6	0.3	0.3	0.1
	Macrophage	0	0	0	0	0	0	0
19/4	**Neutrophil**	0.8	0.5	NA	0.4	0.1	0	0
	Macrophage	0	0	0	0	0	0	0
20/14	**Neutrophil**	0.7	2.4	0.9	0.4	1.1	0.7	0.2
	Macrophage	0	1.2	0	0.4	0	0	0
21/42	**Neutrophil**	1.8	2	0.1	0.6	0.7	0.9	0.2
	Macrophage	0	0	0	0	0.2	0	0
22/48	**Neutrophil**	4.7	1.3	0.5	0.3	0.7	0.9	0
	Macrophage	0	0	0	0	0	0	0

23/17	**Neutrophil**	1.3	0.2	0.6	0.3	0.3	0.2	0.1
	Macrophage	0	0	0	0	0	0	0
26/1	**Neutrophil**	0.5	0.6	0.1	0.4	0	0	0
	Macrophage	0	0	0	0	0	0	0
28/38	**Neutrophil**	0.8	1.6	0.7	1.1	0.5	1.4	0.7
	Macrophage	0	0	0	0.1	0	0	0
29/5	**Neutrophil**	2	0.8	0.3	0.4	0.3	0.9	0.2
	Macrophage	0	0	0	0	0	0	0.2

Neutrophil granulocytes were detected in all dogs (21/21). Their number was not affected by the treatment with azithromycin (P=0.3); however, the number of neutrophil granulocytes reduced over time (P=0.0005) (Table 13). Sixteen (16/21) dogs had neutrophil granulocytes in their ear canals on day 56; out of those, nine (9/12) were and seven (7/9) were not treated with azithromycin. Treatment with methylprednisolone (P=0.2) or fluoroquinolone antibiotics (P=0.7) had no effect on the presence of neutrophil granulocytes in the ear canal.

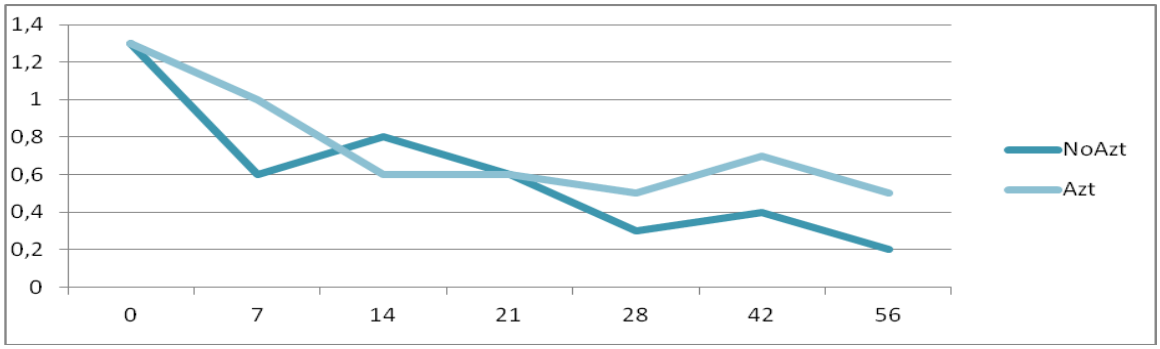
Table 13: Ear cytology (No./OIF) - Neutrophil granulocytes*

Ear cytology (No./OIF) - *Neutrophil* **granulocytes***							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
NoAzt	1.3 ±0.5	0.6 ±0.1	0.8 ±0.4	0.6 ±0.1	0.3 ±0.1	0.4 ±0.1	0.2 ±0.1
Azt	1.3 ±0.3	1.0 ±0.2	0.6 ±0.1	0.6 ±0.1	0.5 ±0.1	0.7 ±0.1	0.5 ±0.2

Legend table 13: data expressed as mean ± SE. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). *Significant effect of time. No./OIF: No. (number) / OIF (Oil Immersion Field) - 1000x magnification.

3.8.5. *Ear cytology evaluation by graph (Neutrophil granulocytes)*

Figure 7: Neurophil granulocyte.



NoAzt: **not treated with** azithromycin **(n=9). Azt: treated with** azithromycin **(n=12).** OIF (Oil Immersion Field) - 1000x magnification. (vertical, average *Neutrophil*, NO. /OIF-horizontal, time). Data expressed as ± SEM.

Macrophages were detected in two (2/21) cytology samples: two (2/9) from the group not treated with azithromycin and neither (0/12) from the group treated with azithromycin. Their number was not affected by the treatment with azithromycin (P=0.7) nor it changed over time (P=0.7) (Table 14). Two dogs (2/21) had macrophages in their ear canal on day 56; both dogs (2/9) were from the group that was not treated with azithromycin. Treatment with methylprednisolone (P=0.3) or fluoroquinolone antibiotics (P=0.5) had no effect on the presence of macrophages in the ear canal.

Table 14: Ear cytology (No./OIF) - Macrophages

Ear cytology (No./OIF) - *Macrophages*							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
NoAzt	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Azt	0. 0	0.0	0.0	0.0	0.0	0.0	0.0

Legend table 14: data expressed as mean ± SE. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). No./OIF: No. (number) / OIF (Oil Immersion Field) - 1000x magnification.

3.9. Measurements of AHLs)

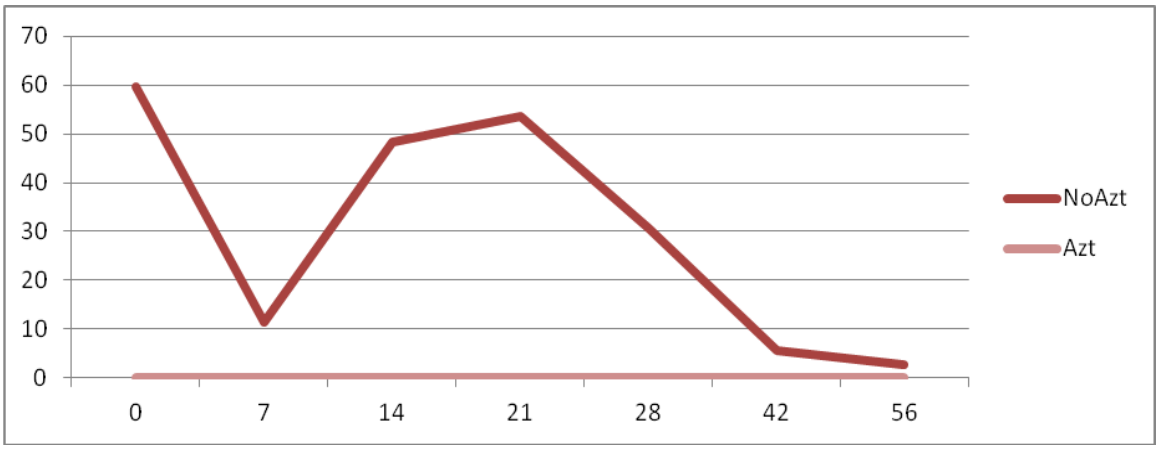
3.9.1. *C4-HSL (BHL)*

Table 15: BHLs measurements by week

Case number	**First visit Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Last visit Week 8**
1/37	0	6.7	6.84	0	0	0	0
2/12	10.84	3.09	0	0	31.25	0	0
3/19	0	5.26	11.47	46.76	0	0	0
4/20	33.73	26.7	29.41	15.26	1.25	2.72	20.72
5/13	0	8.16	1.94	21.24	32.4	3.11	3.41
6/50	0	5.93	1.95	0.81	1.53	1.82	6.85
8/37	14.43	4.1	2.91	14.79	7.76	28.52	5
11/47	0	0	ND	17.65	3.85	3.6	0
12/122	0	2.89	4.46	2.89	3.23	0	6.37
15/26	4.94	7.72	8.04	13.03	ND	6.8	11.04
16/46	59.99	0	ND	12.35	10.72	3.61	0
18/14	53.42	7.66	7.27	11.32	0	0	0
19/4	38.96	6.04	2.94	5.4	0	0	0
20/14	34.37	4.42	0	8.15	72.16	63.88	0
21/42	14.85	3.84	ND	79.94	69.13	60.76	0
22/48	0	0	2.39	2.75	2.23	2.54	0
23/17	0	0	0	0	0	0	0
26/1	433.67	42.96	11.24	382.59	51.41	0	0
28/38	404.31	151.97	3.72	16	8.48	11.89	21.12
29/5	0	3.91	3,02	0	2.6	9.27	14.15

3.9.2. *C4-HSL (BHL) - graphic explanation*

Figure 8: BHL (ng/30mL)



NoAzt**:** not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). BHL: N-butyryl homoserine lactone. Data expressed as ± SEM.

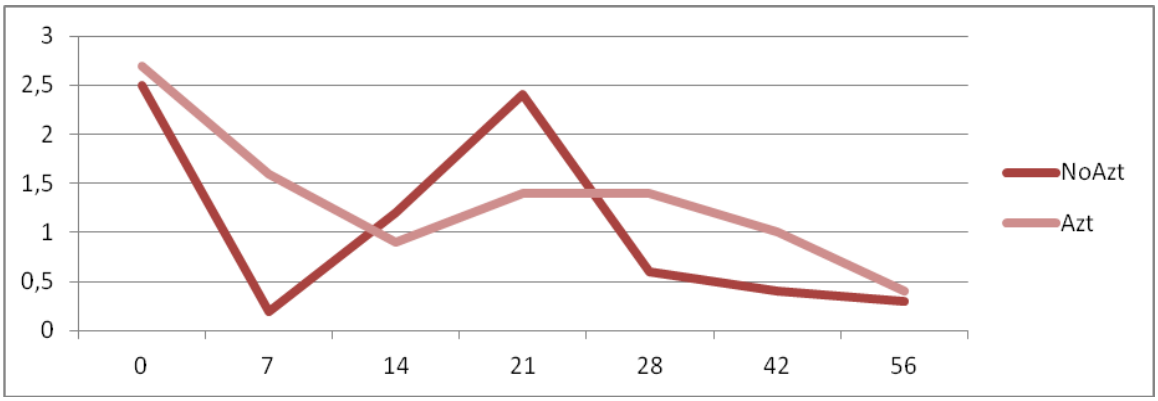
3.9.3. *C6-HSL (HHL)*

Table 16: HHLs measurements by week

Case	First visit						Last visit
1/37	0	0	0	0	0.58	0	0
2/12	0	0	0	0	0.85	0	0
3/19	0	0	0	0	0	0	0
4/20	1.07	1.07	1.21	1.21	0	0	0.51
5/13	0	0	0	1.62	1.53	0	0
6/50	0	0	0	0	0	0	0
8/37	0	0	0	0.56	0	1.01	0.3
11/47	0	0	ND	3.47	0	0	0
12/122	0	0	2.74	0	0	0	0
15/26	0	3.12	1.61	3.24	ND	0	0
16/46	3.84	0	ND	0	3.01	0	0
18/14	4.41	2.84	3.01	3.35	0	0	0
19/4	4.13	2.85	0	0	0	0	0
20/14	0	0	0	0	4.11	4.7	0
21/42	0	0	ND	5.01	4.05	3.95	0
22/48	0	0	0	0	0	0	0
23/17	0	0	0	0	0	0	0
26/1	20.23	1.03	0.13	16.08	3.03	0	0
28/38	19.32	8.77	0	3.47	3.39	3.4	3.82
29/5	0	0	0	0	0	2.5	2.84

3.9.4. *C6-HSL (HHL) - graphic explanation*

Figure 9: HHL (ng/30mL)



NoAzt**:** not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). HHL: N-(hexanoy1)-L-homo- serine lactone. Data expressed as ± SEM.

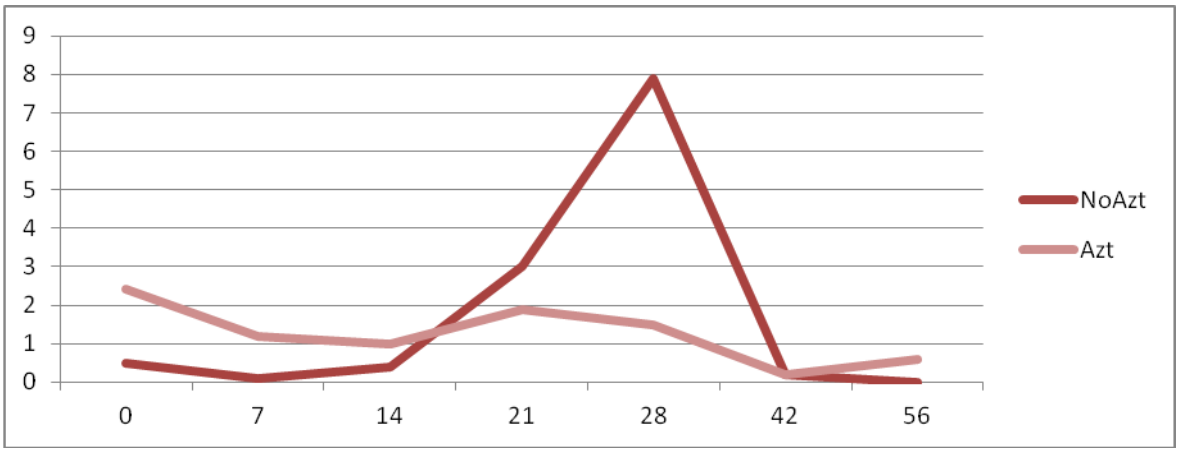
3.9.5. *3-oxo-c12-HSL (OdDHL)*

Table 17: OdDHL measurements by week

Case number	First visit Week 0	Week 1	Week 2	Week 3	Week 4	Week 6	Last visit Week 8
1/37	0	0	0	0	0	0	0
2/12	0	0	0	0	0	0	0
3/19	0	0	0	0	0	0	0
4/20	0	0	0	0	0	0	0
5/13	0	0	0	0.79	0.31	0.14	0
6/50	0	8.67	1.23	0.35	0	0	0
8/37	0	0	0	0	0	0	0
11/47	0	1.3	ND	6.53	1.7	1.19	0
12/122	0	1.31	3.08	1.79	1.06	0	6.29
15/26	0	0	0	0	ND	0	0
16/46	9.36	1.3	ND	7.41	12.25	1.48	1.17
18/14	11.75	1.89	3.25	4.3	0	0	0
19/4	5.22	1.14	1.27	1.39	0	0	0
20/14	0	0	0	0	1.17	1.48	0
21/42	0	0	ND	7.43	1.87	0	0
22/48	0	0	1.15	0	0	0	0
23/17	0	0	0	0	0	0	0
26/1	4.26	0	0	19.77	13.34	0	0
28/38	2.24	0	0	0	0	0	0
29/5	0	0	0	0	0	0	0

3.9.6. *3-oxo-C12-HSL (OdDHL)- graphic explanation*

Figure 10: OdDHL (ng/30mL)



NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). OdDHL: N-(3-oxododecanoyl)-. L-homoserine lactone. Data expressed as ± SEM.

Twelve dogs (12/21) had BHL present in their initial ear canal samples: three (3/9) from the group not treated with azithromycin and nine (9/12) from the group treated with azithromycin. Seven (7/21) dogs had BHL present in their last ear canal samples on day 56; out of those, five (5/12) were and two (2/9) were not treated with azithromycin. Treatment with azithromycin had no effect on BHL (P=0.6). Similarly, time had no effect on the reduction of BHL in the ear canal (P=0.2). Treatment with methylprednisolone (P=0.9) or fluoroquinolone antibiotics (P=0.9) had no effect on the presence of BHL in ear canal samples (Table 18).

A significant association of BHL with rod-shaped bacteria was found indicating that a BHL increase by one unit corresponds to a decrease of 0.2 (±0.08) rod-shaped bacteria /OIF (P=0.01). This was not influenced by azithromycin (P=0.7), methylprednisolone (P=0.9) or fluoroquinolone antibiotics (P=0.2)(Table 18).

Concentration of BHL had no effect on cocci (P=0.2), the overall clinical score (P=0.5) and neutrophil granulocytes (P=0.3). There was no influence of azithromycin, methylprednisolone or fluoroquinolone antibiotics on the effect of BHL on cocci, the overall clinical score and neutrophil granulocytes (P>0.05).

Seven dogs (7/21) had HHL present in their initial ear canal samples: two (2/9) from the group not treated with azithromycin and five (5/12) from the group treated with azithromycin. Four (4/21) dogs had HHL present in their last ear canal samples on day 56; out of those, two (2/12) were and two (2/9) were not treated with azithromycin. Treatment with azithromycin had no effect on HHL (P=0.8). Similarly, time had no effect on the reduction of HHL in the ear canal (P=0.2). Treatment with methylprednisolone (P=0.6) or fluoroquinolone antibiotics (P=0.9) had no effect on the presence of HHL in ear canal samples (Table 18).

A significant association of HHL with rod-shaped bacteria was found indicating that a HHL increase by one unit corresponds to a decrease of 5.2 (±0.08) rod-shaped bacteria /OIF (P=0.008). This was not influenced by azithromycin (P=0.6), methylprednisolone (P=0.9) or fluoroquinolone antibiotics (P=0.3)(Table 18).

Concentration of HHL had no effect on cocci (P=0.2), the overall clinical score (P=0.3) and neutrophil granulocytes (P=0.3). There was no influence of azithromycin, methylprednisolone or fluoroquinolone antibiotics on the effect of HHL on cocci, the overall clinical score and neutrophil granulocytes (P>0.05).

Five dogs (5/21) had OdDHL present in their initial ear canal samples: one (1/9) from the group not treated with azithromycin and four (4/12) from the group treated with azithromycin. Two (2/21) dogs had OdDHL present in their last ear canal samples on day 56, both from the group that was treated with azithromycin. Treatment with azithromycin had no effect (P=0.6) on OdDHL. Similarly, time had no effect on the reduction of OdDHL in the ear canal (P=0.2). Treatment with methylprednisolone (P=0.6) or fluoroquinolone antibiotics (P=0.9) had no effect on the presence of OdDHL in ear canal samples (Table 18).

Concentration of OdDHL had no effect on rod-shaped bacteria (P=0.8), cocci (P=0.2), the overall clinical score (P=0.5) and neutrophil granulocytes (P=0.9). There was no influence of azithromycin, methylprednisolone or fluoroquinolone antibiotics on the effect of HHL on cocci, the overall clinical score and neutrophil granulocytes (P>0.05).

Table 18: N-Acyl homoserine lactones (ng/30mL)

N-Acyl homoserine lactones (ng/30mL)							
Day	**0**	**7**	**14**	**21**	**28**	**42**	**56**
BHL[@]							
NoAzt	59.6 ±47.7	11.5 ±5.1	48.2 ±37.5	53.7 ±41.4	30.7 ±19.4	5.6 ±3.3	**2.8 ±1.7**
Azt	0. 0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	**0.0 ±0.0**
HHL[@]							
NoAzt	2.5 ±2.2	0.2 ±0.1	1.2 ±1.1	2.4 ±1.8	0.6 ±0.4	0.4 ±0.3	**0.3 ±0.3**
Azt	2.7 ±1.6	1.6 ±0.8	0.9 ±0.4	1.4 ±0.5	1.4 ±0.5	1.0 ±0.5	**0.4 ±0.3**
OdDHL							
NoAzt	0.5 ±0.5	0.1 ±0.1	0.4 ±0.3	3.0 ±2.2	7.9 ±6.2	0.2 ±0.1	**0.0 ±0.0**
Az	**2.4 ±1.2**	**1.2 ±0.7**	**1.0 ±0.4**	**1.9 ±0.8**	**1.5 ±1.0**	**0.2 ±0.2**	**0.6 ±0.5**

Legend table 18: data expressed as mean ± SE. NoAzt: not treated with azithromycin (n=9). Azt: treated with azithromycin (n=12). BHL: N-butyryl homoserine lactone. HHL: N-(hexanoy1)-L-homo- serine lactone. OdDHL: N-(3-oxododecanoyl)-. L-homoserine lactone. A significant effect on rod-shaped bacteria.

4. DISCUSSION

This study investigates the influence of azithromycin on *Pseudomonas aeruginosa's* N-acyl homoserine lactones in canine otitis. This study has shown that *P. aeruginosa* has expressed its virulence factors in dogs with otitis via the QS phenomenon. The detection of *P .aeruginosa*-specific AHLs in high concentrations showed that this bacterium produced AHLs in the case of otitis externa. It was not shown that treatment with azithromycin increased the likelihood for *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones. Time as a variable had a significant effect on the reduction of rod-shaped bacteria in the ear canal.

In most cases, a medical approach to the treatment of bacterial diseases is to use antibiotics. Antibiotics are ideally chosen according to isolation of bacteria followed by sensitivity tests. Effective dosage for killing bacteria or inhibiting growth of bacterial cells is chosen (107). When treatment fails due to antibiotic resistance, another approach should be followed. Antibiotic resistance is not a new problem. The first clinical examples were described shortly after the introduction of sulphonamides in 1935 and penicillin in 1941. *P. aeruginosa* is capable of tolerating antibacterial therapy by using the Qs phenomenon and the production of N-AHLs. By reviewing scientific literature, it became clear that if someone could stop or reduce the production of N-AHLs and consequently inhibit the quorum sensing phenomenon (108,109,110,111,112,113), they could probably solve one of the reasons for *P.aeruginosa's* antibiotic resistance.

In our study, a laboratory method for the detection and measurement of *P. aeruginosa's* N-AHLs (BHL, HHL and OdDHL) was designed according to literature data (71,114). The influence of azithromycin on the production of *P. aeruginosa's* N-AHLs, clinical features, cytology and bacterial culture was evaluated using sensitivity tests and biochemistry. The discussion will follow below according to the parts mentioned above.

4.1. Clinical observation

Otitis externa is an inflammation of the ear canal which may result from causes divided into the following groups: predisposing, primary, secondary and perpetuating (1). By checking breeds and history of the dogs that were included in the study, it was established that some of them had hairy concave pinnae (38 %), inbred stenotic ear canal (33 %), *pendulous pinnae* (33 %) and were living in the climate with high humidity (71 %). The mentioned predisposing factors can increase the risk of otitis or enhance the development of the disease (13,115). The mean age of dogs that were included in this study was 8.85 years. Increased age with the weakness of the immune system can predispose to or perpetuate an ear canal inflammation (1,13,116). Based on some researches, the natural killer cell activity, lymphocyte subset distributions, antibody production and mitogen-induced lymphoproliferative responses have all demonstrated age-related changes. Natural killer cells are not significantly affected by age, but the lymphocyte subset analysis revealed a significant age-relation. An age-related decrease has also been observed in the percentage of B-cells concomitant with the increases in T-cell percentages (116).

Many skin diseases may act as a primary trigger for otitis and subsequent *Pseudomonas* infection (17). Among them, allergic dermatitis is the most common and can initiate as many as 43 % of OE cases (18)**.** It changes the balance of physiological secretions and microflora of the ear canal resulting in opportunistic infections. Our study detected eleven cases (52.4 %) with symptoms suggestive for hypersensitivity disorder; 2 cases (2/11) were intradermally tested and had positive results.

We scored clinical signs quantitatively and four variables, namely erythema, stenosis, oedema and pain were evaluated during the study. They were analysed as an overall clinical variable to minimize the effect of individual clinicians' clinical estimates and to minimize the possible deviation in animal presentation (Figure 3).

4.2. Effect of different treatments on clinical signs

Among various recommendations made by experts in the veterinary field, topical use of antimicrobial products including antiseptics seems a simple and effective choice in surface skin multiresistant bacterial infections. In addition to clinical efficacy, local antimicrobial treatment limits the spread of bacteria, thus reducing the need for last-resort broad-spectrum systemic antibiotics which are the main cause of the spread of antibiotic-resistant bacteria.

Methylprednisolone is a potent anti-inflammatory agent (4) and it is required for immune function as an inhibitor for synthesis of almost all known cytokines and cell surface molecules (5). It also reduces the ability of dendritic cells to present antigen and activate T cells suppressing the cellular immune response through the inhibition of IL-12 synthesis and suppression of Th-1 response (117). It was a good choice that we included the agent as a part of our treatment protocol in cases with hypersensitivity symptoms. Our study showed that treatment with methylprednisolone had no effect on three out of four quantitative clinical items (erythema, oedema and stenosis). It showed a significant effect on pain. Treatment with methylprednisolone was statistically analyzed to evaluate its potential effect on the outcome of all of the investigated parameters and should not be interpreted as a function that would show the specific effect on any particular clinical signs. Methylprednisolone did not influence the outcome of the treatment. The resolution of clinical signs was similar between dogs that were or were not treated with methylprednisolone. Based on these findings, treatment with methylprednisolon did not cause the reduction of clinical signs.

Macrolide antibiotic, azithromycin (CP-62,993; 9-deoxo-9a-methyl-9a-aza-9a-homoerythromycin), is a potent drug against gram-negative organisms; it can be compared with erythromycin, while retaining the classic erythromycin spectrum (98). Azithromycin can be the preferred antibiotic used in the treatment of uncomplicated otitis cases (118); however, it should not be used against *P. aeruginosa* because of its known primary resistence to macrolides (119). We have therefore used azithromycin explicitely as an inhibitor of the QS phenomenon (112). Azithromycin is known to reduce the production of several virulence factors of *P .aeruginosa*, such as elastase and rhamnolipids (113). Low concentrations of azithromycin

inhibit the mRNA expression of *N*-acyl homoserine lactone synthesis enzymes, upstream of *lasI* or *rhlI* in *P. aeruginosa* (108). Based on this knowledge, we used topical azithromycin to see its effect on QS in *P. aeruginosa*-infected ears. In the group treated with azithromycin, a significant effect on ear canal clinical parameters was not seen (Graph 3). Time (evaluated at 56 days of the treatment) had a significant effect on each of the clinical signs individually ($P \leq 0.005$). Severity of overall clinical signs also diminished over time ($P < 0.0001$). This result can be attributed to the effect of Tris-EDTA/chlorhexidine solution.

4.3. Culture and isolation of microorganisms

P. aeruginosa and *S. pseudintermedius* are known to be involved in a majority of chronic canine otitis cases. These species can be isolated from more than 70 % of all cases (19). On the first visit, we found a concomitant infection with *P. aeruginosa* and *S. pseudintermedius* in 10 cases (47 %). In five cases (23 %), we found two types of *P. aeruginosa* based on the microbiological examination. Isolated colonies differed on the basis of their morphological features and antibiotic resistance, as well. In 80 % of the cases, we found different types of *Pseudomonas* after the third week of treatment and a high concentration of AHLs was measured during that exact time. This finding may show the activation of Qs, gene transcription and the expression of the new group of genes.

4.4. Antibiotic susceptibility results

Thirty-three percent of isolated *Pseudomonas sp*. were found to be resistant to multiple antibiotics on the initial bacterial culture (*in vitro*). In 38 % of the cases, the resistant *Pseudomonas sp.* was isolated from both groups - the azithromycin and the placebo group on day 56. Treatment with azithromycin and fluoroquinolones did not show any influence on *P. aeruginosa* to develop antibiotic resistance against fluoroquinolones on day 56. The same results were found for the use of methylprednisolone. These findings indicate the importance of performing culture and sensitivity testing before prescribing any antibiotics.

4.5. Bacterial culture and sensitivity

Pseudomonas-associated otitis externa was confirmed in all the included cases at the beginning of the study. *P. aeruginosa* was still isolated from ear canals of 47.6 % of dogs on day 56. Treatment with azithromycin did not influence the presence of *P. aeruginosa* in the ear canal on day 56 of the study nor was the likelihood of *P. aeruginosa*-presence in the ear canal on day 56 of the study influenced by the treatment with methylprednisolone or fluoroquinolone antibiotics.

Presence of rod-shaped bacteria other than *P. aeruginosa* in the ear canal was also assessed. Nine dogs (42.8 %) had rod-shaped bacteria present in their ear canals on day 56. *Proteus sp., E. coli* and *Corynebacterium sp*. were isolated from the cultures. Mixed infections or contamination of culture caused difficulties of judgment and interpretation of the results. By applying statistical methods, we found out that treatment with fluoroquinolone antibiotics did not influence the presence of other rod-shaped bacteria on day 56 of the study nor was the likelihood of the presence of other rods at the end of the study influenced by the treatment with methylprednisolone .

When bacterial culture became negative for the presence of *P. aeruginosa*, other species of rod-shaped bacteria were able to colonize ear canals. In cases where rod-shaped bacteria were detected on the cytological smear, but no growth was present on the bacterial culture, the positive cytology result was attributed to the presence of dead bacteria on the cytological smear.

4.6. Cytology of microorganisms and defense cells

As mentioned before, cytology examination of discharge does not usually make a definitive diagnosis, but it is valuable in determining what infectious agents are present (1,120). To achieve more accurate results, it should be combined with culture (22). By applying cytology techniques, it is possible to check the progress of treatment. The advantage of this technique is also access to rapid results at low cost. Cytological evaluation of otic exudates provides useful

information on the participation of yeast overgrowth (121,122,123). Our study quantitatively assessed the presence of rod-shaped bacteria, cocci, yeasts, neutrophil granulocytes and macrophages. Rods are not considered to be a part of a normal canine ear canal flora; therefore, any presence of rods in the ear canal should be considered pathologic (100). Both groups (the azithromycin and the placebo group) had rod-shaped bacteria in their ear canals on the first day of treatment. Topical application of azithromycin had no effect, whereas time had a significant effect on the reduction of rod-shaped bacteria in the ear canal ($P<0.0001$) (Figure 5). This can be attributed to the effect of Tris-EDTA/chlorhexidine solution. At the end of the treatment, 66.7 % of cases were found to have rod-shaped bacteria in their ear canals. Our study also showed that methylprednisolone or fluoroquinolone antibiotics had no effect on rod-shaped bacteria in the ear canals.

At the beginning of the study, cocci were found in the ear canal of 57 % of dogs. At the end of the study, 33 % of cases had cocci in their ear canals (Figure 6). Our finding shows that treatment with azithromycin and time had no effect on cocci in the ear canal. Methylprednisolone did not show any effect on cocci, whereas treatment with fluoroquinolone antibiotics significantly affected cocci in the ear canal. This was expected since cocci are usually sensitive to fluoroquinolones and are not likely to produce resistance quickly.

Countings of epithelial cells were not included in our analysis because of their unpredictable presence in the clinical sample (21). The mean number of squamous cells was shown to be similar in both, normal and pathological samples (123,21).

M. pachydermatis is a commensal yeast of canine skin and is commonly isolated from normal and inflamed ear canals (124). *M. pachydermatis* can be cultured, but the most accurate way to diagnose the yeasts overgrowth is by making a cytological examination of ear canal exudates (122). Some studies indicate that <10 yeast organisms per high power field (HPF, ×400) should not be considered important because ears of clinically normal dogs can have low numbers of yeast organisms. This number equates to approximately ≥4 yeast organisms per oil immersion field (OIF,×1000) (125). Other researchers have considered two or three *Malassezia* yeast cells per epithelial cell clump or <10 yeast organisms per OIF as normal (123,126,127). Based on

recent studies (21,100), the mean number of inflammatory cells and microorganisms per OIF was calculated for the purpose of our study. Equal or more than 2 yeasts per OIF were considered abnormal. One case had an abnormal number of yeasts at the beginning of the study and the rest were recognised with a normal number of yeasts at the end of the study. Treatment with azithromycin (P=0.2) and time (P=0.5) did not influence the presence of yeasts in the ear canal. Treatment with methylprednisolone or fluoroquinolone antibiotics had no effect on the presence of yeasts in the ear canal.

Inflammatory cells or phagocytosis should not be seen in cured ears' cytology smears (21), although some exceptions were reported (100). Neutrophil granulocytes were detected in all cases at the beginning of the study. Their number was not affected by the treatment with azithromycin (P=0.3); however, the number of neutrophil granulocytes reduced over time (P=0.0005) and was followed by the reduction of cocci (Figure 6,7). Our results show that resolutions of cytological indicators were not influenced by fluoroquinolones nor were they influenced by the treatment with methylprednisolone. The reduction of neutrophills' numbers can be connected to the reduction of numbers of cocci. The presence of neutrophill granulocytes in cases without present bacteria could be attributed to unresolved underlying cause of OE.

Macrophages have long been held to play a key role in tissue repair (128). Azithromycin alters the overall macrophage phenotype. Azithromycin-treated macrophages (mouse macrophage cell line) have demonstrated a significantly reduced production of pro-inflammatory cytokines IL-12 and IL-6 and increased production of anti-inflammatory cytokine IL-10. A decrease in the ratio of IL-12 to IL-10 was also expressed by 60 %. As a result, azithromycin affects an inflammatory process at the level of macrophages and shifts macrophage polarization towards alternatively activated phenotype (129). In response to cytokines and microbial products, mononuclear phagocytes express specialized and polarized functional properties (130,131). We detected macrophages in cytology samples from two (2/21) dogs: two (2/9) from the group not treated with azithromycin and zero (0/12) from the group consecutively treated with azithromycin. Their number was not affected by the treatment with azithromycin (P=0.7) nor it changed over

time (P=0.7). Treatment with methylprednisolone (P=0.3) or fluoroquinolone antibiotics (P=0.5) had no effect on the presence of macrophages in the ear canal.

4.7. N-Acyl homoserine lactones detection and quantification

Autoinducers are produced and active in very low concentrations. AHLs that are produced by different bacterial species differ in length (4-18 carbon atoms) and substitution in C-3 of the molecule acyl side chain (35). Methods for their detection are based on bacterial biosensor strains or chemical methods are employed such as thin –layer chromatography (TLC) (132) and Mass spectrometry (MS) (133). Using QS blockades as a strategy for enhancing host defences against bacterial pathogens is a new field and was one of our research purposes.

P. aeruginosa uses QS as a mean to optimize virulence gene expression and host colonization (7). Due to the involvement of bacterial QS in pathologically relevant events such as symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation, QS inhibitors have the potential to be used in antimicrobial therapy as an adjuvant (49). QS has been shown to be involved in the development of tolerance to various antimicrobial treatments and immunomodulation (8).

Recently, the QS signal molecules (BHL, HHL and OdDHL) isolated from *P. aeruginosa* were found to possess the ability to modulate the immune response. The study was done on human *in vitro* lung tissue with cystic fibrosis. The immune modulatory activity of P*seudomonas* quorum-sensing signals (PQS) suggests that an evaluation of its synthetic analogues may provide better understanding of the structural components that influence its activity and may lead to the development of novel therapeutic agents (134,135). PQS have multiple effects on eukaryotic cells, particularly on those involved in host immunity. PQS inhibits the production of interleukin-2 (IL-2) when human T cells are activated via the T-cell receptor and CD28 (136). OdDHL also inhibits cell proliferation and IL-2 release following mitogen stimulation (137). QS in *P. aeruginosa* consists of two main pairs of LuxI/LuxR homologues termed LasI/LasR and RhlI/RhlR. Similarly to the LuxI, both LasI and RhlI are the autoinducer synthase enzymes, and the resulting complexes of both LasR-autoinducer and RhlR-autoinducer activate a transcription

of various target genes including genes controlling virulence production and formation of biofilms (138).

We found BHLs in 57 % of cases at the beginning of the study. In 33 % of samples, BHLs were still found on day 56. Azithromycin did not show any effect on BHL production. Similarly, time had no effect on the reduction of BHL in the ear canal. Treatment with methylprednisolone or fluoroquinolone antibiotics had no effect on the presence of BHL in ear canal samples.

A significant association of BHL with rod-shaped bacteria was found indicating that BHL increase by one unit corresponds to a decrease of 0.2 (±0.08) rod-shaped bacteria/OIF (P=0.01). This was not influenced by azithromycin (P=0.7), methylprednisolone (P=0.9) or fluoroquinolone antibiotics (P=0.2). This association can also be seen on figure 8 and can be attributed to panic production of BHLs by *P. aeruginosa*, whose numbers were reduced by Tris-EDTA/chlorhexidine treatment.

The concentration of BHL had no effect on cocci (P=0.2), the overall clinical score (P=0.5) and neutrophil granulocytes (P=0.3). There was no influence of azithromycin, methylprednisolone or fluoroquinolone antibiotics on the effect of BHL on cocci, the overall clinical score and neutrophill granulocytes (P>0.05).

HHLs were found in 33 % of dogs in their initial ear canal samples. 19 % still had HHLs in their samples on day 56. Azithromycin had no effect on HHLs. The same results were found for the time item related to HHLs investigated. Treatment with methylprednisolone or fluoroquinolone antibiotics did not show any effect on the presence of HHLs in ear canal samples. The concentration of HHLs had no effect on cocci, the overall clinical score and neutrophil granulocytes. We found a significant association of HHLs with rod-shaped bacteria. HHLs increase by one unit corresponded to a decrease of 5.2 (±0.08) rod-shaped bacteria per OIF. We attribute this correlation to the so-called panic production of bacteria being treated with Tris-EDTA/chlorhexidine. When their number started to get lower, the production of AHLs increased enormously.

In 23 % of cases, OdDHL was found in the first sampling. In 9.5 % of cases, OdDHL was detected in samples on day 56. Treatment with azithromycin had no effect on OdDHL. Also the time item had no effect on the reduction of OdDHL. Concentration of OdDHL did not change the overall clinical score, rod-shaped bacteria, cocci and neutrophil granulocytes .

Amongst three AHLs measured in our study, the most frequently measured was BHL. Nevertheless, two of them (BHLs and HHLs) showed negative correlation to the number of rod-shaped bacteria. We were unfortunately not able to show any influence of AHLs on the overall clinical score.

Since treatment of chronic *Pseudomonas*-associated otitis can be difficult in the manner of the eradication of pathogen, QS antagonists could contribute to a successful therapy. Azithromycin might gain importance in the therapy of chronic ear infections in the future.

5. CONCLUSIONS

1. This study has shown that *Pseudomonas aeruginosa* expresses its virulence factors in dogs with otitis via the *quorum sensing* phenomenon. The detection of *P. aeruginosa*-specific AHLs in high concentrations and the changing of this amount during the treatment supports our research hypothesis.

2. Treatment with azithromycin did not significantly attenuate the synthesis of AHLs in dogs with otitis and a significant reduction of the treatment duration was not established.

3. Amongst three AHLs measured in our study, most frequently measured was BHL. We were not able to show any influence of AHLs on the overall clinical score.

4. Treatment with methylprednisolone and treatment with fluoroquinolone antibiotics both showed a significant beneficial effect on pain, but not on the rest of the four measured clinical signs (erythema, oedema and stenosis).
5. Treatment with fluoroquinolone antibiotics significantly affected cocci in the ear canal. Since many of OE cases deal with mixed infections, this finding supports the use of fluoroquinolone antibiotics, chosen on the basis of a sensitivity test, in complicated canine OE.
6. Thirty-three percent of isolated *P. aeruginosa* were found to be resistant to multiple antibiotics on the initial bacterial culture. This finding indicates the importance of performing culture and sensitivity testing before prescribing any antibiotics.

6. SUMMARY

As a number of other pathogens, *Pseudomonas aeruginosa* controls much of its virulence arsenal by means of extracellular signal molecules in a process termed *quorum sensing* (QS). Using QS blockers as a strategy for enhancing host defenses against bacterial pathogens is a new approach in human and veterinary medicine. The use of QS signal blockers to attenuate bacterial pathogenicity, rather than bacterial growth, is therefore highly attractive, particularly with respect to emergence of multi-antibiotic resistant bacteria.

We designed our trial for the evaluation of topical azithromycin administration effects on N-acyl homoserine lactones produced by *P. aeruginosa*. A hundred and thirty-five ear-lavage samples were collected from 21 dogs with *P. aeruginosa*-associated otitis. Treatment protocol was designed based on clinical observation as well as on cytological and parasitological examination. Dogs were evaluated on days 0, 7, 14, 21, 28, 42 and 56. Clinical signs (like pain, oedema, erythema and stenosis of the ear canal) were evaluated and scored from 0 to 4. Dogs were randomized into two groups: dogs from one group were treated with Tris-EDTA/chlorhexidine solution (Otodine^{R}) and azitromycine and dogs from the other group were treated topically with Tris-EDTA/chlorhexidine solution and placebo (water saline). This was a double blind study. Concentrations of three main AHLs, C4-HSL(BHL), C6-HSL (HHL) and 3-oxo-c12-HSL (OdDHL) were analyzed in ear-lavage samples to assess their influence on other selected variables.

A statistical analysis was made only for numerical outcomes with enough variability (rod-shaped bacteria, cocci, overall clinical score and neutrophil granulocytes).

Twenty-one dogs (mean age 8.85 years, mean weight 20.23 kg, of different breeds and both sexes) were included in this study. Fourteen dogs (14/21) were treated with methylprednisolone. Fifteen dogs (15/21) were treated with fluoroquinolone antibiotics (enrofloxacin or ciprofloxacin

orally). All were topically treated with Tris-EDTA/chlorhexidine solution. Nine dogs were treated topically with azithromycin and twelve topically with placebo.

Time had a significant effect on the treatment outcome. Treatment with methylprednisolone only showed a significant effect on pain. Treatment with fluoroquinolone had no effect on variables in this study. Treatment with azithromycin, Tris-EDTA/chlorhexidine methylprednisolone or fluoroquinolone antibiotics did not show any significant effect on the BHL production.

At the beginning of the study, BHLs were found in 57 % of cases. In 33 % of cases, BHLs were still found at the end of the study (day 56). A significant association of BHLs with rod-shaped bacteria was found indicating that a BHL concentration increase by one unit corresponded to a decrease of 0.2 (±0.08) rod-shaped bacteria/OIF. A comparable result was found for HHLs; an increase by one unit corresponded to a decrease of 5.2 (±0.08) rod-shaped bacteria/OIF. HHLs were found in 33 % of dogs at the beginning of the study and 19 % of cases still had HHLs in their samples at the end of the study (day 56). Treatment with azithromycin, Tris-EDTA/chlorhexidine, methylprednisolone or fluoroquinolone antibiotics did not show any significant effect on HHL production. In 23 % of cases, OdDHLs were found at the beginning of the study and in 9.5 % of cases OdDHLs were detected at the end of the study (day 56). Treatment with azithromycin, Tris-EDTA/chlorhexidine, methylprednisolone or fluoroquinolone antibiotics did not show any significant effects on OdDHL production.

In conclusion, this study did not show beneficial effects of adding azithromycin to the treatment regimen for the treatment of *P. aeruginosa* otits in dogs. Regardless of that, targeting the QS may provide a novel strategy for combating bacterial infections because as it is also evident from this study, it plays an important role in the pathogenesis of *Pseudomonas*-associated otitis.

7. POVZETEK

Bakterije vrste *Pseudomonas aeruginosa*, podobno kot mnogi drugi patogeni mikroorganizmi, izražajo faktorje virulence s pomočjo topnih sporočilnih molekul v procesu, ki ga imenujemo *quorum sensing* (QS). Uporaba blokatorjev QS, da bi izboljšali obrambne mehanizme organizma pred bakterijsko okužbo, pomeni novo strategijo zdravljenja v humani in veterinarski medicini. Blokatorji QS se uporabijo predvsem, da bi se zmanjšala patogenost bakterij in takšen pristop je pomemben zlasti glede na porast okužb z večkratno odpornimi bakterijami.

Namen naše raziskave je bil preučevanje vpliva topikalne aplikacije azitromicina na proizvodnjo N-acyl homoserin laktonov med okužbo sluhovodov psov z bakterijo *P. aeruginosa*. Enaindvajsetim psom z vnetjem zunanjega sluhovoda, povzročenega z bakterijo *P. aeruginosa*, smo odvzeli 135 izpirkov zunanjih sluhovodov, 131 brisov zunanjih sluhovodov za citološko preiskavo, 59 vzorcev krvi in 40 ostružkov kože za parazitološko preiskavo. Vzorce smo zbirali od zime 2010 do pomladi 2012. Protokol obravnave je zajemal klinični pregled, citološke, parazitološke in hematološke preiskave. Sistemsko zdravljenje je temeljilo na antibiotikih, izbranih na podlagi bakteriološke preiskave z antibiogramom. Vzorčili smo naslednje dni v obdobju dveh mesecev: 0., 7., 14., 21., 28., 42. in 56 dan. Klinični znaki vnetja (bolečina, oteklina, rdečina in zožitev sluhovoda) so bili ovrednoteni s številko od 0 do 4 glede na izraženost. Pri vseh psih, vključenih v raziskavo, je bilo ugotovljeno vnetje zunanjega sluhovoda, povzročeno z bakterijo *P. aeruginosa.* Pse smo dvojno slepo razdelili v dve skupini: psi prve skupine so bili zdravljeni z antiseptično raztopino Tris-EDTA/klorheksidin (Otodine^{R}) in azitromicinom, psi druge skupine pa so bili zdravljeni z antiseptično raztopino Tris-EDTA/klorheksidin (Otodine^{R}) in placebom (fiziološka raztopina) lokalno. Petnajst psov (15/21) je bilo zdravljenih s fluorokinoloni (enrofloksacin ali ciprofloksacin) oralno. Štirinajst psov (14/21) je bilo zdravljenih z metilprednizolonom oralno. V izpirkih zunanjega sluhovoda smo

merili koncentracije treh vrst AHL-jev, C4-HSL(BHL), C6-HSL (HHL) in 3-oxo-c12-HSL (OdDHL), ter statistično vrednotili njihov vpliv na druge izbrane spremenljivke.

Statistično smo vrednotili le tiste rezultate, ki so bili dovolj variabilni: število paličastih bakterij, število kokov, skupni klinični parameter (bolečina, oteklina, rdečina in zožitev sluhovoda, vrednoteni skupaj kot ena spremenljivka) ter število nevtrofilnih granulocitov. Podatke smo analizirali z modelom mešanih učinkov. Predpostavljene razlike med rezultati zdravljenja in časa so bile vrednotene s kontrastno analizo. Statistična značilnost je bila postavljena pri $p \leq 0,05$.

V raziskavo smo vključili 21 psov, povprečne starosti 8,85 leta, povprečne teže 20,23 kg, različnih pasem in obeh spolov. Štirinajst psov (14/21) je bilo zdravljenih z metilprednizolonom, petnajst (15/21) pa s fluorokinoloni (enrofloksacin oziroma ciprofloksacin) peroralno. Vsi so bili topikalno zdravljeni s Tris-EDTA/klorheksidin antiseptično raztopino. Devet psov je bilo lokalno zdravljenih s placebom (fiziološka raztopina), dvanajst psov pa je bilo lokalno zdravljenih z azitromicinom.

Pri enajstih psih (52,4 %) smo ugotovili klinične znake, ki so nakazovali alergijsko reakcijo kot primarni vzrok kroničnega vnetja sluhovodov. Zdravljenje z metilprednizolonom ni vplivalo na tri od štirih kvantitativnih kliničnih parametrov (rdečina, oteklina in zoženje sluhovoda), ugotovili pa smo statistično značilen vpliv na bolečino. V primerjavi s skupino, ki je prejemala placebo, nismo ugotovili vpliva zdravljenja z azitromicinom na klinične parametre vnetja sluhovoda pri psih. Pri vseh psih, vključenih v raziskavo, je bilo ugotovljeno vnetje zunanjega sluhovoda, povzročeno z bakterijo *P. aeruginosa. P. aeruginosa* smo iz zunanjega sluhovoda izolirali na koncu raziskave (56. dan) še pri 47,6 % psov. Zdravljenje z azitromicinom na prisotnost bakterije *P. aeruginosa* v sluhovodih ob koncu raziskave ni vplivalo, kot tudi ni vplivalo zdravljenje z metilprednizolonom ali fluorokinoloni. Hkrati s *P. aeruginosa* smo v nekaterih vzorcih izolirali še druge paličaste

bakterije (*Proteus sp., E. Coli* in *Corynebacterium sp*.). Tako zdravljenje s fluorokinoloni kot zdravljenje z metilprednizolonom nista vplivala na prisotnost paličastih bakterij v sluhovodih ob koncu raziskave (56. dan).

P. aeruginosa in *S. pseudintermedius* sta bila v sluhovodih hkrati prisotna v 10/21 (47 %) primerov. Na podlagi mikrobiološke preiskave smo ugotavljali vpletenost dveh tipov bakterije *P. aeruginosa* v 5/21 (23 %) primerov. Pri sedmih psih (33,3 %) smo v začetku raziskave iz sluhovodov izolirali rezistenten sev bakterije *P. aeruginosa*. V 38 % primerov je bil rezistenten sev bakterije *P. aeruginosa* izoliran na koncu raziskave (56. dan). Tako zdravljenje z azitromicinom kot zdravljenje s fluorokinoloni ali metilprednizolonom ni imelo vpliva na pojav rezistentnih sevov bakterije *P. aeruginosa.*

Zdravljenje s Tris-EDTA/klorheksidin 0,15% antiseptično raztopino vsakih 12 ur je statistično značilno vplivalo na zmanjšanje števila paličastih bakterij v zunanjem sluhovodu psov. V naši raziskavi nismo opazili nobenih stranskih učinkov tega zdravljenja. Zdravljenje z 1 ml azitromicina v koncentraciji 50 µg/ml enkrat dnevno lokalno ni vplivalo na število paličastih bakterij v zunanjem sluhovodu psov. Zdravljenje z metilprednizolonom v odmerku 0,5 mg/kg vsakih 24 ur pet dni zapored, nato 0,5 mg/kg vsakih 48 ur ni vplivalo na število paličastih bakterij v sluhovodih psov. Zdravljenje s kinoloni (enrofloksacin oziroma ciprofloksacin v odmerku 5–10 mg/kg enkrat dnevno peroralno) ni vplivalo na število paličastih bakterij v sluhovodih psov. Zdravljenje s Tris-EDTA/chlorhexidin antiseptično raztopino, azitromicinom in metilprednizolonom ni vplivalo na število kokov v sluhovodih psov, na število kokov pa smo ugotovili statistično značilen vpliv zdravljenja s kinoloni. Pri štirih psih (19 %) smo ugotovili prisotnost kvasovk v zunanjih sluhovodih. Prisotnost kvasovk je nihala in smo jo občasno ugotavljali pri različnih psih v različnih časovnih obdobjih. Zdravljenje s Tris-EDTA/klorheksidin antiseptično raztopino, azitromicinom, metilprednizolonom ali kinoloni ni vplivalo na število kvasovk v sluhovodih psov.

Prisotnost nevtrofilnih granulocitov smo ugotovili v zunanjih sluhovodih vseh psov na začetku raziskave. Na njihovo število zdravljenje z azitromicinom, metilprednizolonom in kinoloni ni značilno vplivalo. Ugotovili pa smo statistično značilno zmanjšanje števila nevtrofilnih granulocitov s časom, hkrati z zmanjšanjem števila kokov. Ta učinek pripisujemo vplivu zdravljenja s Tris-EDTA/klorheksidin antiseptično raztopino. Makrofage smo ugotovili pri dveh (9,5 %) psih. Zdravljenje s Tris-EDTA/klorheksidin antiseptično raztopino, azitromicinom, metilprednizolonom ali kinoloni ni vplivalo na število makrofagov v sluhovodih psov.

Ob začetku raziskave smo dokazali prisotnost sporočilnih molekul tipa BHL v 57 % vzorcev izpirkov zunanjih sluhovodov. V 33 % primerov smo BHL sporočilne molekule dokazali ob koncu raziskave (56. dan). Zdravljenje s Tris-EDTA/klorheksidin antiseptično raztopino, azitromicinom, metilprednizolonom ali kinoloni ni statistično značilno vplivalo na proizvodnjo sporočilnih molekul tipa BHL pri okužbi zunanjih sluhovodov psov z bakterijo *P. aeruginosa.* Ugotovili pa smo statistično značilno povezavo BHL sporočilnih molekul s številom paličastih bakterij v sluhovodih psov: povečanje koncentracije BHL za eno enoto je ustrezalo zmanjšanju 0,2 (±0,08) paličastih bakterij v vidnem polju mikroskopa pri veliki povečavi (1000-kratna povečava). Primerljiv rezultat smo ugotovili tudi za sporočilne molekule tipa HHL: povečanje koncentracije HHL za eno enoto je ustrezalo zmanjšanju 5,2 (±0,08) paličastih bakterij v vidnem polju mikroskopa pri veliki povečavi (1000-kratna povečava). Sporočilne molekule tipa HHL smo sicer ugotovili pri 33 % vzorcev na začetku raziskave in pri 19 % vzorcev na koncu raziskave (56. dan). Zdravljenje z antiseptično raztopino Tris-EDTA/klorheksidin, azitromicinom, metilprednizolonom ali kinoloni ni statistično značilno vplivalo na proizvodnjo sporočilnih molekul tipa HHL pri okužbi zunanjih sluhovodov psov z bakterijo *P. aeruginosa.* Sporočilne molekule tipa OdDHL smo ugotovili v 23 % vzorcev na začetku razislave in pri 9,5 % vzorcev na koncu raziskave (56. dan). Zdravljenje s Tris-EDTA/klorheksidin antiseptično raztopino, azitromicinom, metilprednizolonom ali kinoloni ni statistično značilno vplivalo na proizvodnjo sporočilnih molekul tipa OdDHL pri okužbi zunanjih sluhovodov psov z bakterijo *P. aeruginosa.*

Mehanizem QS je vpleten v patogenezo okužb zunanjih sluhovodov psov z bakterijo *P. aeruginosa*. Odkrivanje sporočilnih molekul QS v zgodnjem obdobju bolezni in uporaba blokatorjev QS pri zdravljenju obeta uspešnejše zdravljenje bakterijskih okužb v prihodnosti.

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10. APPENDIX

10.1. Owner's consent

Številka protokola

PRISTANEK LASTNIKA ŽIVALI ZA SODELOVANJE V RAZISKAVI

Podpisani_____,

naslov_____, se strinjam, da je pes /psica z imenom_____,

pasme_____, rojen/a_____, vključen/a v raziskavo zdravljenja gnojnega vnetja ušes pri psih. Seznanjen/a sem z namenom in eventuelnimi tveganji raziskave. S tem se obvezujem, da bom v času raziskave, ki traja 2 meseca, dosledno spoštoval/a navodila raziskovalcev doc.dr.Tine Kotnik, dr.vet.med., doc.dr.Modesta Vengušta, dr.vet med. in Javida Hosseinija Tabrizija, dr.vet.med.

Če bi nastale okoliščine, ki bi lahko vplivale na jemanje predpisanega zdravila ali dogovorjeni datum kontrolnega obiska (nezmožnost obiska ob dogovorjenem datumu, nezmožnost dajanja zdravila živali) bom nemudoma poklical enega od raziskovalcev na telefonsko številko spodaj in se posvetoval z njim.

Raziskovalci se obvezujejo, da bodo raziskavo vodili v skladu s splošno veljavnimi etičnimi načeli in v dobro živali in lastnika.

V Ljubljani, dne _____.

Podpis lastnika živali: Podpis raziskovalca:

_____ _____

10.2.

10.3. Clinical observation form

VPRAŠALNIK Št.QS:

(Gnojno vnetje sluhovodov) Datum: / /

Tt: kg

1. Ime in priimek lastnika:

2.Ime živali: 3. Starost: 4. Pasma:

5. Kako dolgo ima vaš pes že vnetje ušes-(a)?

6. Ali se je vnetje ušes pojavilo že kdaj prej? NE DA

7. Če da, kolikokrat se je vnetje pojavilo v zadnjem letu?

8. S katerim zdravilom in v kakšnem odmerku so bila predhodna vnetja ušes zdravljena?

9. Kako dolgo je pes zdravila dobival in v kakšni obliki (tablete, injekcije, kapljice)?

10. Če na vprašanji 8 in 9 ne znate odgovoriti, prosim napišite ime veterinarja, ki je zdravilo predpisal:

11. Ali se kdaj pojavi vnetje kože?

12. Ali ima poleg vnetja ušes (in kože) še kakšne druge težave z zdravjem, kakšne?

13. Prosim naštejte vrste hrane in priboljške, s katerimi hranite vašega psa:

14. Ali je bila zaradi vnetja ušes predpisana dieta in katera?

15. Drugi pomembni podatki:

10.4. Client education (Slovenian & English)

SL: Kako dajemo zdravila?

(Navodila za lastnike psov)

Spoštovani lastnik psa,

veterinarska klinična študija je raziskava, narejena z namenom odgovoriti na določena vprašanja glede učinkovitosti zdravljenja pri živalih. Raziskavo v katero ste vključeni, vodi skupina veterinarjev specialistov na Veterinarski fakulteti v Ljubljani. Za sodelovanje se vam že vnaprej zahvaljujemo in vas prosimo, da si preberete spodnja navodila.

Tablete in kapsule

Gobec psa razklenete tako, da z dlanjo ene roke objamete spodnjo čeljust in se s palcem uprete ob zobe zgornje čeljusti, na drugi strain pa s prsti porivate spodnjo čeljust navzdol. Ko gobec odprete, z drugo roko položite tableto globoko na koren jezika, zaprete gobec in masirate grlo, dokler tablete pes ne pogoltne. Če boste tableto položili na jezik preveč spredaj ali na stran, jo bo pes izpljunil.

To give a pill to your dog, slip your thumb into the space behind one of the dog teeth and press upward on the roof of the mouth. As the mouth begins to open, press down on the lower jaw with the opposite thumb. Alternatively, press in on both lips from above the muzzle. As the skin pushes in behind the canines, the dog will open his mouth. Insert the pill well to the back of the tongue in the middle of the mouth. If you place the pill too far forward or to the side of the tongue, the dog will spit it out. Close the dog's mouth and massage or rub his throat until he swallows. If the dog licks his nose, the pill has been swallowed. You can also give him a syringe full of water to make sure he swallows, or give the dog a small treat after the pill goes down.

EN: How to give ear drops to your dog?

Take out one of the prepared drug tubes from the freezer (you will receive 84 tubes). Keep the tube in your hand and wait for 2 minutes (the tube content changes from solid to liquid and the temperature of liquid will be about your body temperature). Keep the tube out of his sight for now. Hold him the way you normally hold him for the drops and give him a treat if he does not struggle. If he does, try again later - no treats, no cuddles, just ignore the struggling. Once you can hold him without a struggle, start handling his ears. Gently at first, just lifting the flap or moving it around.
Eventually you should be able to handle the tube while holding him. OR have another person handle the bottle while you hold him. Eventually move the uncapped tube to his ear for a second - treat if there is no struggle and ignore him if there is. Ear infections are painful, so your dog is probably just reacting to the pain. If you make the whole experience up until the drops go in LESS stressful and make him think that it is all associated with treats, he may be less stressed up until the drops go in. Eventually THAT will stop as the infection clears up. Remember to do the drops QUICKLY. No putting one drop in, wait a few seconds, put the other in, wait, another, wait, etc. It should be drop in, drop in, treat, done.

10.5. Clinical protocol form

QS Research CLINical Protocol Sample No: PQS_____Date:_____

Clinical file label WEEK No.: 0 1 2 3 4 6 8

1. Signed consent from the patient's owner □

2. History and physical examination of the dog □

3. Blood collection □

4. Two cotton swabs from each ear □

5. Ears rinsing with sterile saline (30 ml) and storage on ice

left □ right □

6. Epidermal scrub (from the vertical ear canal)

left □ right □

7. Azithromycin / placebo treatment

left_____ mL right_____ mL

8. Antibiotic therapy: Antimycotic therapy:

a)_____mg/kg/____hrs a)_____mg/kg/____hrs

b)_____mg/kg/____hrs b)_____mg/kg/____hrs

c)_____mg/kg/____hrs c)_____mg/kg/____hrs

9. Change of the diet (see attached protocol) □

10. Allergic tests (final visit):

ID test □ Serum IgE □

10.6. Laboratory protocol form

QS Research LABoratory Protocol Sample No: PQS :_____

Date: _____

Clinical file label WEEK No.: 0 1 2 3 4 6 8

1. Centrifuging and freezing ☐

2. Preparation of slides for cytology ☐

3. Results of parasitological examination

Negative ☐ Positive for_____

10.7. Clinical observation

QS Research CLINical Observation Sample No: PQS: ----------

Clinical file label

WEEK No. Date:		WEEK No. Date:		WEEK No. Date:		WEEK No. Date:	
Foreign bodies		Foreign bodies		Foreign bodies		Foreign bodies	
Ear mites		Ear mites		Ear mites		Ear mites	
Stenosis		Stenosis		Stenosis		Stenosis	
Ruptured ear drum		Ruptured ear drum		Ruptured ear drum		Ruptured ear drum	
Erythema		Erythema		Erythema		Erythema	
Oedema		Oedema		Oedema		Oedema	
Pain		Pain		Pain		Pain	

Legend: Y=yes, N=no, 1=mild, 2=moderate,3=severe,4=very severe

10.8. Antibiotic susceptibility results

Table 19: Antibiotic susceptibility results

Set NO:	Ts.	Am	s	cip	s	Cef.	s	Enr.	s	Gen	S.	Pip	s.	sul	s	az	s	tob	s	Pol	s	mar	s	tic	s	im	s
1/37	Ps	14	R	16	R	21	S	16	R	8	R	28	S	6	R	6	R	18	S	18	S	20	S	-	-	28	S
1/37	L	17	S	19	I	20	I	19	R	15	S	29	S	6	R	6	R	18	S	18	S	10	R	-	-	28	S
2/12	PS	26	S	27	S	15	I	12	R	24	S	25	S	6	R	6	R	28	S	18	S	16	I	-	-	39	S
2/12	L	28	S	29	S	17	I	13	R	25	S	25	S	6	R	6	R	27	S	18	S	16	I	15	S	40	S
3/19	PS	30	S	20	I	6	R	6	R	26	S	25	S	6	R	6	R	31	S	18	S	6	R	-	-	30	S
3/19	L	27	S	20	S	18	I	6	R	20	S	27	S	6	R	6	R	24	S	17	S	6	R	21	S	30	S
4/20	PS	25	S	34	S	21	S	21	S	22	S	30	S	6	R	6	R	26	S	18	S	27	S	22	S	32	S
4/20	L	6	R	12	R	8	R	18	I	6	R	36	S	35	S	6	R	6	R	17	S	13	R	37	S	28	S
4/20	LB	22	S	29	S	19	I	18	I	18	S	27	S	6	R	6	R	23	S	18	S	20	S	21	S	27	S
5/13	PS	24	S	32	S	16	I	24	S	23	S	27	S	6	R	6	R	26	S	16	S	27	S	22	S	30	S
5/13	L	23	S	22	S	20	I	-	-	21	S	28	S	6	R	6	R	-	-	18	S	14	R	28	S	25	S
5/13	LB	26	S	24	S	21	S	15	R	22	S	29	S	6	R	6	R	28	S	18	S	17	I	27	S	26	S
6/50	PS	24	S	32	S	16	I	24	S	23	S	27	S	6	R	6	R	26	S	16	S	27	S	22	S	30	S
6/50	L	22	S	28	S	18	I	no	-	19	S	29	S	6	R	6	R	24	S	18	S	19	S	22	S	17	S
6/50	LB	20	S	27	S	16	I	no	-	19	S	29	S	6	R	6	R	23	S	18	S	17	I	22	S	16	S
8/37	PS	22	S	24	S	14	I	10	R	19	S	20	S	6	R	6	R	24	S	19	S	9	R	15	S	24	S
8/37	L	26	S	20	S	11	R	10	R	21	S	21	S	6	R	6	R	25	S	20	S	11	R	6	R	24	S
8/37	LB	25	S	24	S	15	I	10	R	20	S	26	S	6	R	6	R	26	S	18	S	16	I	23	S	27	S
11/47	PS	23	S	40	S	20	I	24	S	17	S	25	S	6	R	6	R	22	S	19	S	26	S	20	S	19	S
11/47	L	30	S	13	R	21	S	6	R	25	S	32	S	-	-	-	-	19	S	19	S	6	S	26	S	27	S
12/12	PS	21	S	31	S	22	S	21	S	17	S	27	S	6	R	6	R	23	S	20	S	25	S	25	S	27	S
12/12	L	22	S	27	S	23	S	17	I	18	S	13	I	-	-	-	-	27	S	22	S	21	S	22	S	10	R
12/12	LB	30	S	39	S	26	S	28	S	26	S	31	S	6	R	6	R	28	S	20	S	30	S	26	S	29	S

Set NO:	Ts.	Am	s	cip	s	Cef.	s	Enr.	s	Gen	S.	Pip	s.	sul	s	az	s	tob	s	Pol	s	mar	s	tic	s	im	s
15/26	PS	28	S	36	S	25	S	27	S	24	S	30	S	10	R	6	R	27	S	19	S	28	S	26	S	28	S
15/26	L	25	s	19	I	18	I	6	R	19	S	32	S	-	-	-	-	25	S	15	S	6	R	25	S	24	S
16/46	PS	23	S	40	S	20	I	24	S	17	S	25	S	6	R	6	R	22	S	19	S	26	S	20	S	19	S
16/46	L	26	S	35	S	23	S	24	S	25	S	33	S	-	-	-	-	25	S	20	S	27	S	23	S	27	S
18/14	PS	23	S	33	S	19	I	22	S	23	S	29	S	-	-	-	-	24	S	-	-	28	S	-	-	-	-
18/14	L	24	S	30	S	18	I	20	S	20	S	30	S	-	-	-	-	25	S	18	S	23	S	20	S	28	S
19/4	P	23	S	33	S	19	I	22	S	23	S	29	S	-	-	-	-	24	S	21	S	28	S	-	-	-	-
19/4	L	25	S	20	I	20	I	17	I	20	S	30	S	-	-	-	-	27	S	20	S	10	I	16	S	27	S
20/14	PS	23	S	21	S	18	I	11	R	19	S	26	S	6	R	6	R	23	S	19	S	16	I	20	S	22	S
20/14	L	28	S	34	S	26	S	-	-	23	S	26	S	26	S	6	R	28	S	20	S	14	R	20	S	30	S
21/42	PS	23	S	21	S	18	I	11	R	19	S	26	S	-	-	-	-	23	S	19	S	16	I	20	S	22	S
21/42	L	28	S	34	S	26	S	-	-	23	S	33	S	-	-	-	-	13	I	22	S	20	S	27	S	24	S
22/48	PS	25	S	25	S	22	S	11	R	21	S	31	S	6	R	6	R	26	S	20	S	17	I	23	S	22	S
23/17	PS	22	s	30	s	6	R	20	R	20	s	27	s	6	R	6	R	24	s	19	s	23	s	15	s	27	s
26/1	PS	29	S	35	S	24	S	26	S	29	S	35	S	-	-	-	-	24	S	23	S	32	S	23	S	27	S
26/1	L	27	S	29	S	20	I	18	I	25	S	29	S	-	-	-	-	27	S	18	S	18	S	19	S	18	S
28/38	PS	20	S	29	S	16	I	18	I	18	S	25	S	-	-	-	-	24	S	18	S	21	S	19	S	28	S
29/5	PS	26	S	32	S	18	I	24	S	25	S	29	S	-	-	-	-	27	S	19	S	27	S	21	S	26	S

Legend			
	AM=Amikacin	GEN=Gentamicin	R=Resistance
	AZ=Azithromycin	L=last sampling	S=Sensitive
	CIP=Ciprofloxacin	MAR=Marbofloxacin	SUL=Sulfamethoxazole/trimethoprim
	CEF=Ceftriaxone	PIP=Piperacilin	TIC=Ticarcilin
	IM=Imipenem	POl=Polymyxin B	TOB=Tobramycin
	ENR=Enrofloxacin	PS=Primary sampling	TS=time of sampling