Determination of N-acylhomoserine lactones of *Pseudomonas aeruginosa* in clinical samples from dogs with otitis externa

Darja Kušar*, Karin Šrimpf, Petra Isaković, Lina Kalšek, Javid Hosseini, Irena Zdovc, Tina Kotnik, Modest Venguš and Gabrijela Tavčar-Kalcher

**Abstract**

**Background:** Bacterial intercellular communication, called quorum sensing, takes place via the production and collective response to signal molecules. In Gram-negative bacteria, like *Pseudomonas aeruginosa*, these signaling molecules are N-acylhomoserine lactones (AHLs). *P. aeruginosa* is a common cause of inflammation of the ear canal (otitis externa) in dogs. It employs quorum sensing to coordinate the expression of host tissue-damaging factors, which are largely responsible for its virulence. The treatment of *P. aeruginosa*-associated otitis is challenging due to a high intrinsic resistance of *P. aeruginosa* to several antibiotics. Attenuation of quorum sensing signals to inhibit bacterial virulence is a novel strategy for the treatment of resistant bacterial pathogens, including *P. aeruginosa*. Therefore, it is important to recognize and define quorum sensing signal molecules in clinical samples. To date, there are no reports on determination of AHLs in the veterinary clinical samples. The purpose of this study was to validate an analytical procedure for determination of the concentration of AHLs in the ear rinses from dogs with *P. aeruginosa*-associated otitis externa.

Samples were obtained with rinsing the ear canals with physiological saline solution. For validation, samples from healthy dogs were spiked with none or different known amounts of the selected AHLs. With the validated procedure, AHLs were analyzed in the samples taken in weekly intervals from two dogs, receiving a standard treatment for *P. aeruginosa*-associated otitis externa.

**Results:** Validation proved that the procedure enables quantification of AHLs in non-clinical and clinical samples. In addition, a time dependent reduction of AHL concentration was detected for the treated dogs.

**Conclusions:** Our results indicate that liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is superior in detecting AHLs compared to other chromatographic techniques. This is the first report on determination of AHLs in the clinical samples of veterinary importance. The analytical procedure described in this paper is capable of supporting novel antimicrobial strategies, which target quorum sensing.

**Keywords:** Dogs, Otitis externa, Clinical samples, *Pseudomonas aeruginosa*, Quorum sensing, N-acylhomoserine lactones, Liquid chromatography-tandem mass spectrometry, Validation

* Correspondence: darja.kusar@vf.uni-lj.si
Veterinary Faculty, University of Ljubljana, Gerbičeva 6051-1115, Ljubljana, Slovenia

© 2016 The Author(s). Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

*Pseudomonas aeruginosa* is an important opportunistic pathogen of humans and animals, causing severe infections when the immune system is compromised [1, 2], or after long-lasting antibiotic treatments, injuries and medical procedures [3, 4]. *P. aeruginosa* is a Gram-negative bacterium ubiquitously present in the water and soil. It is naturally resistant to penicillin and aminopenicillins, and to the first and second generations of cephalosporins. It is usually susceptible to aminoglycosides, fluoroquinolones, lipopeptides, ureidopenicillins, carboxypenicillins, carbapenems, and cephalosporins of the third generation [15]. The resistance of *P. aeruginosa*, however, can rapidly develop against any antibiotic [5].

Behavior of bacteria is coordinated, at the level of the microbial community, by their gene expression at the intra-species level or less commonly between species. Such coordination of communal behavior is controlled through the production and detection of signal molecules or autoinducers, which is called quorum sensing (QS) [6–10]. The QS system defines the expression of various bacterial phenotypes, depending on the local density of bacterial population, including the virulence factors, biofilm formation and resistance to antibiotics [11]. It is associated with pathogenesis of various diseases in humans and animals, and has a clinically evident effect in human cystic fibrosis (CF) [12], endocarditis and osteomyelitis in rabbits [13, 14], bovine mastitis [15], gastrointestinal diseases [9], and ear canal infections, dermatitis and other secondary infections in dogs [16].

The secondary factors, including *Malassezia* spp. (yeasts), and bacteria of the genera *Staphylococcus*, *Proteus*, *Streptococcus*, and *Pseudomonas*, often cause complications to the inflammation of the ear canal (otitis externa, OE) in dogs [17, 18]. Involvement of *P. aeruginosa* in OE (*P. aeruginosa*-associated OE, PaOE) increases the morbidity, prolongs the treatment, and reduces the possibility of a favorable outcome [19, 20]. The ability of *P. aeruginosa* to produce biofilms and synchronize its virulence through QS is very important for the conservation and/or perpetuation of infection [21]. The long and narrow ear canal of dogs represents a very suitable environment for *P. aeruginosa* colonization and the subsequent activation of its QS [7].

One or several signal molecules may be involved in QS. In Gram-negative bacteria, the most common are N-acylhomoserine lactones (AHLs or AI-1) [9, 10, 22, 23]. *P. aeruginosa* QS system shows a hierarchical arrangement with las system controlling rhl [6–8, 24–28]. It mainly produces N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), whereas production of N-hexanoyl-L-homoserine lactone (C6-HSL) and shorter 3-oxo-HSLs (3-oxo-C6-HSL, 3-oxo-C8-HSL and 3-oxo-C10-HSL) is less abundant [29, 30]. It was shown that 3-oxo-C12-HSL has the most critical role, as it regulates the expression of several genes involved in activation of the subordinate rhl system, where C4-HSL is the most significant autoinducing molecule [6]. In addition to its signalling function, 3-oxo-C12-HSL also exerts antibacterial activities against some other pathogens [31] and a direct immunomodulatory effect on the mammal host [32], itself functioning as a virulence determinant [33] and contributing to the establishment of chronic infections [34]. Under some growth conditions, activation of the las system does not precede the activation of rhl, but just the opposite, indicating the environmental dependence of their hierarchy [35]. There are various layers of sophistication to *P. aeruginosa* AHL-dependent QS [34, 36] and approximately 10 % of *P. aeruginosa* genome is directly or indirectly regulated by this multi-signal QS system [37]. Some genes are las specific, some rhl, and others respond to both 3-oxo-C12-HSL and C4-HSL [37]. In addition, at least four QS signalling mechanisms were reported to comprise *P. aeruginosa* QS network, being modulated in accordance to the environmental cues, also of host origin, influencing the virulence phenotypes of *P. aeruginosa* [36].

Detection of signal molecules associated with QS could serve as an indicator for the severity of infection, success of a treatment, and to estimate the outcome of disease [22]. Disruption of QS, called quorum quenching (QQ) [38], could represent a therapeutic tool, complementing or substituting the antibiotic treatment, which would reduce the risk for the development of antibiotic resistance [39]. It was shown that QQ can be achieved through the enzymatic destruction of QS signals, development of antibodies to QS signal molecules, or via agents which block QS [40]. The inhibitors of QS would reduce bacterial pathogenicity, also by increasing the susceptibility of existing biofilms to antibiotics or phagocytosis, and increase effectiveness of the host immune system [39, 41, 42]. Identifying molecules involved in QS in the clinical samples is, therefore, of great importance.

Several reports describing chemical analytical methods for the identification and quantification of selected AHLs are available. The use of a thin layer chromatography [7, 12]; gas chromatography with mass spectrometry (GC-MS) [43, 44]; high-performance liquid chromatography (HPLC) with UV detection [6], mass spectrometry (MS) [12], or coupled with tandem MS (MS/MS) [22, 45]; nuclear magnetic resonance (NMR) [26]; and colorimetry [46] was reported. In addition, a bacterial biosensor system for the detection of AHLs was also described [9]. Although a number of analytical methods were developed, only a few are applicable for determination of low AHL concentrations in clinical samples [22]. Determination of AHLs in physiological samples or
complex biological matrices is more complicated than
in bacterial culture supernatants due to the interfer-
ence from matrix, causing most of the analytical
problems [7, 9, 12, 22]. The mechanisms behind this
phenomenon, commonly referred to as matrix effects
and leading to signal suppression or enhancement,
are still under investigation due to the great variety
of available matrices and the unpredictable effect they
might have on the final result [47]. Hence, data defining
pathophysiological concentrations of AHLs in the clinical
samples have not been often reported. In picomolar to
micromolar concentrations, AHLs were discovered in the
sputum [7, 12], mucopurulent secretions of respiratory
tract [48], lung tissue of patients with CF [49], and saliva
and faeces of individuals with gastrointestinal disorders
[22]. To the best of our knowledge, no reports were pub-
lished regarding detection of QS signal molecules in veter-
inary clinical samples.

In the present work, the analytical procedure for deter-
mination of AHLs of P. aeruginosa from canine OE
clinical samples was introduced and validated. It consisted
of the extraction of AHLs (3-oxo-C12-HSL, C4-HSL and
C6-HSL) from the ear rinses and their quantification using
the liquid chromatography with tandem mass spectrom-
etry (LC-MS/MS).

Methods
Reagents
Solid standard substances C4-HSL, C6-HSL and 3-oxo-
C12-HSL with certified purity of 97 % (Sigma-Aldrich,
St. Louis, MO, USA) were used. Stock standard solu-
tions of individual AHLs with the concentration of
1 mg/ml (C4-HSL, C6-HSL) and 0.91 mg/ml (3-oxo-
C12-HSL) were prepared in methanol. Mixed working
standard solutions for preparing the calibration curve
containing all the three selected AHL signal molecules,
each at the concentration from approx. 1 to 60 ng/ml,
were prepared in a mixture of methanol and deionized
water (35 + 65). Dichloromethane used for the extrac-
tion of AHLs and methanol used for the mobile phase were
of analytical and chromatography grade purity, respect-
ively (Merck, Darmstadt, Germany). As the mobile
phase, a gradient mixing of methanol and deionized
water (35 + 65). Dichloromethane used for the extraction
of AHLs (3-oxo-C12-HSL, C4-HSL and C6-HSL) from the ear rinses
and their quantification using the liquid chromatography
with tandem mass spectrometry (LC-MS/MS).

Sample collection and preparation
Samples were obtained by rinsing the ear canals of dogs
with saline solution (30 ml). The collected samples were
adjusted to the final volume of 30 ml by sterile saline,
centrifuged at 3100 × g for 30 min at 4 °C, and the super-
natants were stored at −80 °C till extraction.

For validation, the ear rinses from healthy dogs spiked
with known amounts of C4-HSL, C6-HSL and 3-oxo-
C12-HSL were used. None of the samples contained
AHLs in detectable concentrations prior to spiking. An
aliquot of 0.320 ml of the mixed working standard
solution containing AHLs in concentrations of approx.
60 ng/ml (61.5 ng/ml of C4-HSL and C6-HSL, 56.9 ng/ml
of 3-oxo-C12-HSL) was added to 10 ml of the super-
natant, giving the final concentrations of approx. 2 ng/ml
(1.97 ng/ml of C4-HSL and C6-HSL, 1.82 ng/ml of 3-oxo-
C12-HSL). Thus, a 10-ml sample contained approx. 20 ng
each of each AHL (19.7 ng of C4-HSL and C6-HSL, 18.2 ng of
3-oxo-C12-HSL).

To test the validated analytical procedure with clinical
samples, the ear rinses of two PaOE dogs, collected
weekly during the treatment, were used. Samples were
taken during a scheduled veterinary checkup at the pri-
mary clinic or at the university clinic. As a control, the
ear rinse from a healthy dog was employed.

Analytical procedure
For the extraction of AHLs from the ear rinses, a proced-
ure used for determination of AHLs in sputum [7, 12] was
modified. One third (10 ml) of a 30-ml sample was
extracted three times with 10-ml portions of dichloro-
methane. The combined extracts were evaporated to
dryness at 60 °C using the vacuum evaporator (Syncore
Reactor R-48, Büchi, Flawil, Switzerland). The residue
was dissolved in 1 ml of the mixture of methanol and
deionized water (35 + 65).

The particular AHLs were determined by LC-MS/MS
at conditions described elsewhere [22, 45]. In brief, elu-
tion of the column was performed with a mixture of
methanol–water. The mixing was isotropic for 4 min
(35 % methanol, 65 % deionized water), followed by a
linear gradient of methanol concentration from 35 to
95 % in 4 min and then back to 35 % in 1 min. The fol-
lowing 2 min, the column was washed again with the
mixture of methanol and water (35 + 65). The mobile
phase flow rate was 0.25 ml/min, injection volume 10 µl,
and column temperature 30 °C. Detection with MS was
performed in the positive ion mode. Voltage at the capil-
ary was 0.4 kV. The monitored ions and other parame-
ters are given in Table 1. 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 × 100 mm (Agilent, Santa Clara, CA, USA) and mass
selective detector Xevo TQ MS (Waters).

Validation procedure
To perform the linearity test, and to determine the limit
of detection (LOD) and the limit of quantification
(LOQ), the mixed working standard solutions of AHLs
at concentrations from approx. 1 to 60 ng/ml were used, corresponding to AHL concentrations of approx. 0.1 to 6 ng/ml in the ear rinses. The estimation of LOD and LOQ was based on visual evaluation, testing concentrations in the target range expected for AHLs in clinical samples. The LOQ estimate was defined as the lowest amount or concentration of an analyte that is reasonably achievable according to the target range and should be regarded as the reporting limit; LOD was estimated as one third (30 %) of LOQ [50].

The repeatability of results was tested by analyzing five replicates of the ear rinse sample with the addition of C4-HSL, C6-HSL and 3-oxo-C12-HSL at 1.97 ng/ml (C4-HSL, C6-HSL) and 1.82 ng/ml (3-oxo-C12-HSL). The analyses were performed at the same day and by the same operator. For testing the within-laboratory reproducibility, five replicates of the sample were prepared for two more times by two operators at two different days. The results obtained from these experiments were also used for determination of the recovery.

**Statistical procedures**

The mean \( \bar{x} \) for each AHL was calculated. The repeatability and the within-laboratory reproducibility were expressed by standard deviation \( s_r \) and \( s_R \), respectively) and relative standard deviation \( RSD_r \) and \( RSD_R \), respectively). The \( RSD_R \) values were compared to the values calculated from the Horwitz equation \( RSD = 2^{(1 - 0.5 \log C)} \), taken as a reference. The recovery was calculated according to the equation \( \text{rec} = 100 \frac{x}{a} \), where \( a \) represents the spiked concentration of the analyte in the sample [50, 51].

**Culture examination**

Prior to rinsing the ears of PaOE affected dogs with sterile saline, cotton-swab samples were taken from the ears for bacteriological examination. Samples were inoculated onto blood agar plates (Columbia agar; Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 5 % sheep blood and incubated aerobically at 37 °C for 48 ± 2 h. After 24-h and 48-h incubation, plates were examined for the growth of *Pseudomonas* spp. and 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 and colleagues [52]: biochemical characteristics of the isolates were determined using the commercial kit Api 20 NE (BioMérieux, France) according to the manufacturer’s instructions.

**Results**

In Figs. 1, 2 and 3, chromatograms of individual AHL standard solutions C4-HSL (19.7 ng/ml), C6-HSL (19.7 ng/ml) and 3-oxo-C12-HSL (18.2 ng/ml) are presented, respectively. Retention times of C4-HSL, C6-HSL and 3-oxo-C12-HSL were 1.99, 4.76 (4.77) and 9.16 min, respectively (Figs. 1, 2 and 3, Table 1). For each AHL, two transitions of the precursor to product (fragment) ion are shown (e.g., for C4-HSL 172.096 to 101.980 and 172.096 to 144.073). In the figures, the common display of LC-MS/MS chromatograms, with the x-axis showing time and y-axis the relative signal intensity (relative to the highest peak in chromatogram, in %), was adopted.

Equations describing the calibration lines were \( y = 2027x + 708 \) (C4-HSL), \( y = 2603x + 2039 \) (C6-HSL) and \( y = 1594x + 204 \) (3-oxo-C12-HSL). Regression coefficients of the calibration curves were 0.999, 0.998 and 0.999, respectively. Based on the responses obtained, the lowest calibration point (1 ng/ml – or 0.1 ng/ml when referring to 10-ml volumes) was estimated as the lowest quantifiable concentration. Therefore, LOQ for 10-ml samples was estimated to 0.1 ng/ml (1 ng per 10-ml sample) and LOD accordingly lower to 0.03 ng/ml (0.3 ng per 10-ml sample). Expressed in molar concentrations, LOD and LOQ were approx. 175 pM and 584 pM for C4-HSL (with molar mass of 171.2 g/mol), 151 pM and 502 pM for C6-HSL (199.2 g/mol), and 101 pM and 336 pM for 3-oxo-C12-HSL (297.4 g/mol), respectively. Thus, with LOD in the range of 100–200 pM and LOQ in 300–600 pM, LC-MS/MS was confirmed suitable for the purpose as picomolar to micromolar concentrations were reported for clinical samples [7, 12, 22, 48, 49].

### Table 1 LC-MS/MS parameters

| AHL          | Retention time [min] | Precursor ion m/z | Product ion m/z | Cone voltage [V] | Collision cell voltage [V] |
|--------------|----------------------|-------------------|----------------|-----------------|---------------------------|
| C4-HSL       | 1.99                 | 172.096           | 101.980        | 18              | 10                        |
|              |                      | 172.096           | 144.073        | 18              | 8                         |
| C6-HSL       | 4.76 (4.77)          | 200.160           | 101.984        | 18              | 10                        |
|              |                      | 200.160           | 98.986         | 18              | 6                         |
| 3-oxo-C12-HSL| 9.16                 | 298.266           | 102.038        | 26              | 12                        |
|              |                      | 298.266           | 197.138        | 26              | 14                        |

\( m/z: \) mass-to-charge ratio
In Tables 2 and 3, parameters of the repeatability and reproducibility test are given. Recoveries, calculated from the results obtained under the within-laboratory reproducibility conditions, were 54, 89 and 79 % for C4-HSL, C6-HSL and 3-oxo-C12-HSL, respectively. Before calculations, the outliers were excluded using the Grubbs statistical test [50].

When the analytical procedure for determination of C4-HSL, C6-HSL and 3-oxo-C12-HSL was successfully validated, the ear rinses (clinical samples) obtained from a healthy dog (negative control) and two dogs with PaOE (positive samples) were analyzed. In Figs. 4 and 5, a chromatogram of the negative control and an example of C4-HSL chromatogram belonging to a positive sample from the PaOE diseased dog are shown, respectively. The positive-sample chromatogram clearly shows a peak belonging to C4-HSL (Fig. 5), which is missing in the negative-control chromatogram (Fig. 4). It should be recalled that the y-axis scales are in relative units (normalized to the largest peak), resulting in the jagged line reflecting only the background noise in Fig. 4. The largest peak was 7.800 × 10^2 units in Fig. 4 and 5.161 × 10^5 units in Fig. 5, therefore the signal was approx. 1000-times higher in the positive sample than in the negative.

In Table 4, the total masses of C4-HSL, C6-HSL and 3-oxo-C12-HSL determined in the ear rinses of two dogs with PaOE collected at weekly intervals during the treatment (week 0, 1, 2, 3, 4, 6 and 8) are given. The contents of C6-HSL and 3-oxo-C12-HSL were distinctly lower than of C4-HSL, making useful only the measurements of C4-HSL contents. In the first dog, the content of C6-HSL was much lower than of C4-HSL, but roughly correlated to fluctuations of C4-HSL; C6-HSL masses were close to LOQ (1 ng per 10-ml sample; week 0–3), but also below LOQ (week 8) or even LOD (0.3 ng per 10-ml sample; week 4 and 6). Surprisingly, the content of 3-oxo-C12-HSL was always below LOD. In the second dog, the contents of C6-HSL and 3-oxo-C12-HSL were below LOD but reached LOQ (C6-HSL) or LOD (3-oxo-C12-HSL) in the third and fourth week, which was in congruence with the increased C4-HSL levels.

In the first dog, the content of C4-HSL decreased in the first month of treatment, but then slightly increased in the sixth week and markedly in the eighth week of treatment (Table 4). At that time (week 6 and 8), a secondary infection of the ear canal was also observed by culture examination, showing the presence of two different P. aeruginosa strains. In the second dog, the disease management started with C4-HSL below LOD (day 0),
but its content increased after the first week of treatment (Table 4). The time coincided with isolation of *Proteus mirabilis* from the ear swab. In the second week of treatment, the content of C4-HSL decreased and *P. mirabilis* was no longer detected by culture examination. In the third and fourth week, however, a marked increase of C4-HSL content could be observed, with the appearance of additional *P. aeruginosa* strain (already in week 2) and the reappearance of *P. mirabilis*. Later, in the second month of the treatment, the content of C4-HSL decreased and the secondary infection was resolved (week 6 and 8).

**Discussion**

The present study demonstrated that AHL signal molecules can be quantified in the ear rinses of PaOE affected dogs using LC-MS/MS. This method proved superior when compared to other chromatographic techniques that were tested in the scope of our preliminary validation experiments: GC-MS [43, 44] and HPLC with UV detection [6]. Namely, of the three methods selected for determination of AHLs in OE clinical samples only LC-MS/MS enabled determination of signal molecules at target concentrations. Two major (3-oxo-C12-HSL, C4-HSL) and one minor (C6-HSL) AHL of *P. aeruginosa* were monitored. Identification of AHLs in clinical samples can have an important diagnostic, therapeutic and prognostic value in human and veterinary medicine.

The detection and quantification limits of the analytical procedure were low enough (LOD: 0.03 ng/ml; LOQ: 0.1 ng/ml) to enable determination of C4-HSL, C6-HSL and 3-oxo-C12-HSL in the ear rinses from dogs with PaOE. The linearity of the procedure was confirmed with the regression coefficient of the calibration curve higher than 0.995. The precision, repeatability and the within-laboratory reproducibility, of the procedure was expressed by standard deviations $s$ [ng] and relative standard deviations RSD [%]. Two outliers were eliminated based on the Grubbs test [50], both belonging to the 3-oxo-C12-HSL measurements from the same day of validation. The resulting RSD$_R$ values for C4-HSL (35 %), C6-HSL (22 %) and 3-oxo-C12-HSL (20 %) were compared to the reference values derived from the Horwitz equation: for the tested concentration of 2 ng/ml, the reference RSD$_R$ value was 41 % [50, 51]. Since the obtained RSD$_R$ values were below 41 %, the reproducibility was considered satisfactory.

The recoveries of C4-HSL, C6-HSL and 3-oxo-C12-HSL at the concentration of 2 ng/ml were 54, 89 and 79 %, respectively. Regarding requirements for the performance of analytical procedures (e.g., Decision 2002/657/EC [51]), the recoveries of C6-HSL and 3-oxo-C12-HSL were considered satisfactory; whereas the recovery of C4-HSL (54 %) was suboptimal (it should be 70–110 %). Similar recovery values were reported previously [7]. The suboptimal recovery of C4-HSL should not be considered a significant limiting factor, because the analytical procedure was primarily used for monitoring the changes in AHL concentrations with time and not to determine their absolute values. However, the obtained recovery values can be used for the correction of results to achieve a good approximation of the actual contents in a sample whenever appropriate.

**Table 2** Parameters obtained within the repeatability test

| Replicate | C4-HSL | C6-HSL | 3-oxo-C12-HSL |
|-----------|--------|--------|---------------|
| Average mass [ng] in 10-ml sample | 11.3 | 19.9 | 15.6 |
| $s_r$ [ng] | 5.7 | 2.2 | 2.8 |
| RSD$_r$ [%] | 50.2 | 11 | 18 |

**Table 3** Parameters obtained within the reproducibility test

| Replicate | C4-HSL | C6-HSL | 3-oxo-C12-HSL |
|-----------|--------|--------|---------------|
| Average mass [ng] in 10-ml sample | 10.6 | 17.5 | 14.3 |
| $s_R$ [ng] | 3.7 | 3.8 | 2.9 |
| RSD$_R$ [%] | 35 | 22 | 20 |
| Recovery [%] | 54 | 89 | 79 |
Sample sediments, which were obtained after the initial centrifuging of the ear rinses, were tested for residual levels of AHLs prior to validation. This was performed to verify the predominating distribution of analytes into the liquid phase. Sediment analysis was the same as implemented for the supernatants. The quantities of individual AHLs were approximately 20-times lower in the sediments than in their respective supernatants (data not shown), indicating the residual level of less than 5%. Therefore, only supernatants were used for further analysis.

Samples for the present study were collected from two dogs with \textit{Pa}OE, which was confirmed with the routine bacteriological analyses. The treatment regimen was standard [53–58] and included regular ear irrigations with topical saline flush and local administration of Tris-EDTA/chlorhexidine (0.15 % solution; Otodin, Industria Chimica Fine ICF, Palazzo Pignano, Italy) once daily. Namely, the antiseptic solution Tris-EDTA/chlorhexidine was proven beneficial and safe for the \textit{Pa}OE diseased dogs with unlikely selection for bacterial resistance which would predispose the chronic character of the disease [55–58]. A systemic antibiotic (enrofloxacin) treatment was only employed after clinical condition of dogs deteriorated. To avoid confusion, the state of clinical condition should not be directly deduced from the results of culture examination or AHL content in the samples. Enrofloxacin was administered in the last 14 days of treatment to the first dog and in the first 14 days to the second.

Regarding the total masses of AHLs in the ear lavages of dogs with \textit{Pa}OE, the content of C6-HSL was in general much lower than of C4-HSL, but the fluctuations of these two roughly correlated. The content of C6-HSL was approximating LOQ or even LOD. Moreover, the content of 3-oxo-C12-HSL was always below LOQ or, in most cases, below LOD. According to the results, only C4-HSL measurements showed applicable value. In the first dog, results showed that the content of C4-HSL decreased in the first month of Tris-EDTA/chlorhexidine treatment in accordance to expectations [58], but increased again toward the end of the treatment (slightly in week 6 and markedly in week 8). The increase could be assigned to a secondary infection of the ear canal,
which was confirmed by culture examination showing the presence of an additional \textit{P. aeruginosa} strain.

In the second dog, the content of C4-HSL was below LOD at the beginning of the disease management, but increased after the first week of treatment. \textit{P. mirabilis}, a Gram-negative opportunistic pathogen ubiquitously present in the environment and a common secondary factor of OE in dogs [17, 18], was also isolated from the ear canal. Since Gram-negative bacteria employ AHLs for quorum communication, cross-talk communication between the two species cannot be excluded. The inter-species communication was suggested previously between \textit{P. aeruginosa} and \textit{P. mirabilis}, since a variation of AHL QS system was observed in \textit{P. mirabilis} with signaling molecules being structurally similar to AHLs [59]. Namely, in both the cell-free culture supernatants of \textit{P. aeruginosa} and of \textit{P. mirabilis}, compounds capable of activating the AHL biosensor were found, not belonging to AHLs but to structurally similar diketopiperazines (DKPs). In concentration-dependent manner, DKPs were able to activate the AHL biosensor, although their physiological role remains to be discovered, but also suggesting the existence of cross talk among bacterial signaling systems. However, the QS system in \textit{Proteus} sp. is not completely understood and its signaling molecules are not well defined. Although signaling molecules with the structure identical to AHLs are not produced by \textit{P. mirabilis} [60], it was shown that \textit{P. mirabilis} reacts and changes the population features when AHLs produced by other Gram-negative bacteria are added to the culture media [61]. The content of C4-HSL in the second dog decreased in the second week of treatment, which coincided with the disappearance of \textit{P. mirabilis} from the bacteriological sample. The marked increase in C4-HSL was detected in the third and fourth week, which coincided with an additional strain of \textit{P. aeruginosa} and the reappearance of \textit{P. mirabilis} in the bacteriological sample. After the fourth week, the C4-HSL content declined and \textit{P. mirabilis} was no longer present. By the end of the treatment course, the additional strain of \textit{P. aeruginosa} also disappeared.

No AHLs were detected in the negative control sample from a healthy dog. The analytical procedure described and validated in this study is, therefore, capable of monitoring changes in AHL concentration throughout the treatment regimen, reflecting the state of \textit{Pseudomonas} infection.

Concerning 3-oxo-C12-HSL, low concentrations were detected in this study. Similar was observed previously in samples from human patients with CF [7].

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Day & Week & 1st dog & & Culture examination & \\
& & & AHL mass [ng] & & \\
& & & C4-HSL & C6-HSL & 3-oxo-C12-HSL & \\
& & & P. aeruginosa & Other bacteria & \\
0 & 0 & 33.7 & 1.1 & <0.3 & + & – \\
7 & 1 & 26.7 & 1.1 & <0.3 & + & – \\
14 & 2 & 29.4 & 1.2 & <0.3 & + & – \\
21 & 3 & 15.3 & 1.2 & <0.3 & + & – \\
28 & 4 & 1.3 & <0.3 & <0.3 & + (two strains) & – \\
42 & 6 & 2.7 & <0.3 & <0.3 & + (two strains) & – \\
56 & 8 & 20.7 & <1 & <0.3 & + (two strains) & – \\
\hline
Day & Week & 2nd dog & & Culture examination & \\
& & & AHL mass [ng] & & \\
& & & C4-HSL & C6-HSL & 3-oxo-C12-HSL & \\
& & & P. aeruginosa & Other bacteria & \\
0 & 0 & <0.3 & <0.3 & <0.3 & + & – \\
7 & 1 & 8.2 & <0.3 & <0.3 & + & \textit{Proteus mirabilis} \\
14 & 2 & 1.9 & <0.3 & <0.3 & + (two strains) & – \\
21 & 3 & 21.2 & 1.6 & <1 & + (two strains) & \textit{Proteus mirabilis} \\
28 & 4 & 32.4 & 1.5 & <1 & + (two strains) & \textit{Proteus mirabilis} \\
42 & 6 & 3.1 & <0.3 & <0.3 & + (two strains) & – \\
56 & 8 & 3.4 & <0.3 & <0.3 & + & – \\
\hline
\end{tabular}
\caption{Total masses of AHLs in the ear lavages of dogs with PaOE (\textit{Pseudomonas aeruginosa}-associated OE), sampled in the course of treatment}
\label{table:ahl}
\end{table}

\textsuperscript{a}Results of culture examination are given for better understanding and are explained in the text.

\textsuperscript{b}Indicating a secondary infection with a different \textit{P. aeruginosa} strain, deteriorating the clinical condition of dogs and causing the increase of AHL content in the infected ear canal.
interconnected or independent, reasons for this analytical outcome are possible: i) 3-oxo-C12-HSL is not a major AHL present in the ear canals of PaOE-diseased dogs or in clinical samples in general; ii) deficiency of one or more P. aeruginosa QS components leading to reduced synthesis/depolyment of 3-oxo-C12-HSL; iii) the in-vitro results, which are presently the gold standard for understanding the biology of QS and AHLs, are different from the in-vivo behavior of P. aeruginosa QS; and iv) limitations of the membrane transport, which are selective to effusion of 3-oxo-C12-HSL from P. aeruginosa cytoplasm.

Regarding the results obtained in this study and previously for the samples from human CF patients [7], it is possible that 3-oxo-C12-HSL is not a major AHL present in clinical samples, disease-related or in general. One should not be misled by the terminology that the rhl system is ‘only’ subordinate to las, as we can speculate that rhl might be more adaptable to current conditions in its milieu or could support greater part of QS communication. A deficiency of one or more QS components of the rhl system was previously reported for P. aeruginosa canine OE isolates [62], and P. aeruginosa mutants with the impaired las were also obtained from clinical samples [63]. These QS deficient strains are regularly isolated from clinical or environmental samples and are called the ‘cheating’ individuals, profiting from the benefits of the cooperating population without contributing to the community [64]. Not all P. aeruginosa strains/isolates produce both C4-HSL and 3-oxo-C12-HSL [7, 63]. In addition to the QS deficiencies, the expression of QS is different in P. aeruginosa isolates from clinical samples or grown as biofilms when compared to those that are grown in laboratory as broth cultures in nutrient-rich media [12, 65]. Broth cultures are being intensively studied to explain the composition and hierarchy of bacterial QS systems. In the natural environments, however, biofilms are predominating over the planktonic growth [7, 12]. P. aeruginosa is notorious for its ability to form biofilms [10]. Isolated from CF sputum of human patients, P. aeruginosa produces C4-HSL in excess of 3-oxo-C12-HSL when grown in biofilms but not in broth cultures [65]. If the higher content of C4-HSL over 3-oxo-C12-HSL observed in the present study is due to variation in their production, P. aeruginosa from the ear canals of PaOE-diseased dogs is also predominantly present as biofilms. Another reason for low concentrations of 3-oxo-C12-HSL in the studied clinical samples, in addition to QS deficiencies of P. aeruginosa strains and differential gene expression in biofilms, could be the fact that 3-oxo-C12-HSL requires an active membrane transport (pump) for its efflux from the cells to the surroundings, whereas C4-HSL diffusion has no such limitation and P. aeruginosa cells are freely permeable to C4-HSL [7, 66].

If P. aeruginosa is predominately growing in biofilms in the ear canals of PaOE-diseased dogs, another explanation for the low amounts of 3-oxo-C12-HSL can be suggested. Namely, AHLs are restricted in diffusion by the extracellular matrix of biofilms and they reach adequate intracellular concentrations for induction of QS-dependent genes at lower values in comparison to planktonic growth phenotype [67]. Therefore, it is possible that less 3-oxo-C12-HSL is needed in biofilms for the activation of the subordinate rhl system, but more with planktonic phenotypes due to diffusion throughout the liquid milieu to reach concentrations high enough for the activation of rhl.

P. aeruginosa can adapt to different environments via its regulatory networks and multifunctional signal molecules [34, 36], which can be monitored using the methods such as the one reported in this study. Disrupting QS regulation is a valuable approach to develop new therapeutic strategies. Implementation of alternative therapies (QQ strategies) for PaOE, or other P. aeruginosa infections, should have a beneficial impact on patients [68, 69]. Plants, for example, represent a large and unexplored pool of potentially QQ bioactive compounds [70, 71].

Conclusions
The QS system is a defining part of the pathologies caused by P. aeruginosa and other bacteria. Detection and quantification of AHLs in clinical samples may provide an important diagnostic and prognostic tool to follow the disease progress, which has already been proven in human CF cases. It would also be possible to initiate and/or terminate, or evaluate, the success of antibiotic treatments in diseases of the PaOE complexity. In this study, the LC-MS/MS analytical procedure for determination of selected P. aeruginosa AHLs (C4-HSL, C6-HSL, 3-oxo-C12-HSL) in clinical samples obtained from PaOE-diseased dogs was introduced and successfully validated. For the clinical samples obtained from two PaOE-diseased dogs, low content of 3-oxo-C12-HSL was observed with C4-HSL predominating. To explain the phenomenon, it was suggested, in addition to other hypotheses, that P. aeruginosa is growing in the infected ear canals organized in biofilms.

Abbreviations
3-oxo-C12-HSL: N-(3-oxododecanoyl)-L-homoserine lactone; AHLs (or AI-1): N-acylhomoserine lactones; C4-HSL: N-butanoyl-L-homoserine lactone; C6-HSL: N-hexanoyl-L-homoserine lactone; CF: Cystic fibrosis; DPK: Diketopiperazines; GC-MS: Gas chromatography with mass spectrometry; HPLC: High-performance liquid chromatography; LC-MS/MS: Liquid chromatography with tandem mass spectrometry; LOD: Limit of detection; LOQ: Limit of quantification; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; NMR: Nuclear magnetic resonance; OE: Otitis externa (inflammation of the external ear canal); PaOE: Pseudomonas aeruginosa-associated OE; QQ: Quorum quenching; QS: Quorum sensing; rec: recovery; RSDr: Relative standard deviation (RSDr for repeatability, RSDs for reproducibility); s: standard deviation (s for repeatability, s2 for reproducibility)
Acknowledgements
This research was conducted in the frame of Research Programs P4-0092 “Animal health, environment and food safety” and P4-0053 “Endocrine, immune and enzyme responses in healthy and sick animals”, funded by the Slovenian Research Agency.

Funding
This study was supported by the Slovenian Research Agency grants P4-0053 and P4-0092. The funder had no role in study design, data collection and analyses, decision to publish or preparation of the manuscript.

Availability of data and materials
All the relevant data is contained within the manuscript. The results for replicates from the repeatability test and the reproducibility test are freely available from the corresponding author.

Authors’ contributions
Study conception and design: DK, MV, GTK; Acquisition of samples: JH, TK, MV; Analytical procedures: KS, PI, LK, JH, GTK; Iz; Interpretation of data: DK, MV, GTK; Drafting of manuscript: DK, MV, GTK; Critical revision: DK, KS, PI, LK, JH, Iz, TK, MV, GTK. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All study procedures complied with the European union and Slovenian Animal Protection Acts and were approved by the Animal ethics committee of the University of Ljubljana (ref #: 34401-19/2010/2). The experimental studies involved client-owned animals, thus an informed consent from the owner was obtained before the dogs entered the study and the studies adhered to a high standard (best practice) of veterinary care.

Received: 25 September 2015 Accepted: 22 September 2016
Published online: 18 October 2016

References
1. Fergie JE, Shema SJ, Lott L, Crawford R, Patrick CC. Pseudomonas aeruginosa bacteremia in immunocompromised children: analysis of factors associated with a poor outcome. J Clin Infect Dis. 1994;18:390–4.
2. Mendelson MH, Gurtman A, Szabo N, Seibert E, Meyers BR, Policar M, Cheung TW, Lillenfeld D, Hammer G, Reddy S, Choi K, Hirschman SZ. Pseudomonas aeruginosa bacteremia in patients with AIDS. Clin Infect Dis. 1994;18:388–92.
3. Brewer SC, Wunderink RG, Jones CB, Leeper KVJ. Ventilator-associated Pseudomonas aeruginosa pneumonia due to Pseudomonas aeruginosa. Chest. 1996;109:1019–29.
4. Weese JS. A review of multidrug resistant surgical site infections. Vet Comp Orthop Traumatol. 2008;21:1–7.
5. Stratte V, Yardosan D. Pseudomonas aeruginosa – a phenomenon of bacterial resistance. J Med Microbiol. 2000;59:1133–48.
6. Reimann C, Beyerle M, Latifi A, Winteler H, Foglino M, Lazdunski A, Stewart GSAB, Bycroft BW. The Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. Mol Microbiol. 1997;24:309–19.
7. Erickson DL, Endersby R, Kirkham S, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG. Pseudomonas aeruginosa quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infect Immun. 2002;70:1783–90.
8. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 2005;21:319–46.
9. Kumari A, Pasini P, Deo SK, Flomenhout T, Shahidhar H, Daunert S. Biosensing systems for the detection of bacterial quorum signaling molecules. Anal Chem. 2006;78:7603–9.
10. Boyer F, Eeckhaut V, Van Immerssle F, Pasmans F, Ducatelle R, Haebersbruck F. Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. Vet Microbiol. 2009;135:187–95.
11. Miller MB, Basler BL. Quorum sensing in bacteria. Annu Rev Microbiol. 2001;55:165–99.
12. Middleton B, Rodgers HC, Carama M, Knox AJ, Williams P, Hardman A. Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum. FEMS Microbiol Lett. 2002;207:1–7.
13. Cheung AL, Eberhardt KJ, Chung E, Yeaman MR, Sullam PM, Ramos M, Bayer AS. Diminished virulence of a sar/aqr- mutant of Staphylococcus aureus in the rabbit model of endocarditis. J Clin Invest. 1994;94:1815–22.
14. Gillaspy AF, Hickman SG, Skinner RA, Thomas JR, Nelson CL, Smeltzer MS. Role of the accessory gene regulator (agr) in pathogenesis of staphylococcal osteomyelitis. Infect Immun. 1995;63:3373–80.
15. Buzzola FR, Alvarez LP, Tuchscherr LPN, Barbagelata MS, Lattar SM, Calvinho L, Sordelli DO. Differential abilities of capsulated and noncapsulated Staphylococcus aureus isolates from diverse agr groups to invade mammary epithelial cells. Infect Immun. 2007;75:886–91.
16. Bannoejhr J, Ben Zakour NL, Waller AS, Guardabassi L, Thoday KL, van den Broek AHM, Fitzgerald JR. Population genetic structure of the Staphylococcus intermedius group: insights into agr diversification and the emergence of methicillin-resistant strains. J Bacteriol. 2007;189:161–2.
17. August JR, Otits externa. A disease of multifactorial etiology. Vet Clin North Am Small Anim Pract. 1988;18:731–42.
18. Paterson S. A review of 200 cases of otitis externa in the dog. Vet Dermatol. 2003;14:249. Abstract.
19. Kiss G, Radvalýi S, Szigeti G. New combination for the therapy of canine otitis externa. Microbiologia of otitis externa. J Small Anim Pract. 1993;38:351–6.
20. Caffotti DN, LeRoy ST. Otitis externa in dog: etiology and clinical findings; literature review and retrospective study of 752 cases. Prat Med Clin Anim Comp. 1997;32:243–57.
21. Kuchma SL, Connolly JP, O’Toole GA. A three-component regulatory system regulates biofilm maturation and type III secretion in Pseudomonas aeruginosa. J Bacteriol. 2005;187:1441–54.
22. Kumari A, Pasini P, Daunert S. Detection of bacterial quorum sensing N-acyl homoserine lactones in clinical samples. Anal Bioanal Chem. 2008;391:1619–27.
23. Manefeld F, Turner SL. Quorum sensing in context: out of molecular biology and into microbial ecology. Microbiology. 2002;148:3762–4.
24. Gambello MJ, Iglewski BH. Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. J Bacteriol. 1991;173:3000–9.
25. Ochsner UA, Koch AK, Fiechter A, Reiser J. Isolation and characterization of a regulatory gene affecting rhodanilid biosurfactant synthesis in Pseudomonas aeruginosa. J Bacteriol. 1994;176:2044–54.
26. Pearson JP, Gray KM, Pasllador L, Tucker KD, Eberhard A, Iglewski BH, Greenberg EP. Structure of the autoinducer required for expression of Pseudomonas aeruginosa virulence genes. Proc Natl Acad Sci U S A. 1994;91:197–201.
27. Pearson JP, Pasllador L, Iglewski BH, Greenberg EP. A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 1995;92:1490–4.
28. Pasllador L, Cook JM, Gambello MJ, Rust L, Iglewski BH. Expression of Pseudomonas aeruginosa virulence genes requires cell-to-cell communication. Science. 1993;260:1127–30.
29. Winson MK, Camara M, Latifi A, Foglini M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW, Ladzunski A, Stewart GSAB, Williams P. Multiple N-acyl-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 1995;92:29427–31.
30. de Kieft TR, Iglewski BH. Bacterial quorum sensing in pathogenic infections. Infect Immun. 2000;68:4839–49.
31. Qazi S, Middleton B, Muhammar SH, Cockeye A, Hill P, O’Shea P, Chhabra SR, Camara M, Williams P. N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in Staphylococcus aureus. Infect Immun. 2006;74:4910–9.
32. Cooley M, Chhabra SR, Williams P. N-acylhomoserine lactone-mediated quorum sensing: a twist in the tail and a blow for host immunity. Chem Biol. 2008;15:1141–7.
33. Telford G, Wheeler D, Williams P, Tomkins PT, Appleby P, Sewell H, Stewart GSB, Bycroft BW, Pitchard DJ. The Pseudomonas aeruginosa quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. Infect Immun. 1998;66:36–42.
34. Williams P, Camara M. Quorum sensing and environment adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol. 2009;12:182–91.
35. Duan K, Surette MG. Environmental regulation of Pseudomonas aeruginosa PAO1 Las and Rhl quorum-sensing systems. J Bacteriol. 2007;189:4827–36.
36. Lee J, Zhang L. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein Cell. 2015;6:26–41.
37. Schuster M, Greenberg PE. A network of networks: quorum-sensing gene regulation in Pseudomonas aeruginosa. Int J Med Microbiol. 2006;296:73–81.
38. Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH. Quenching quorum-sensing dependent bacterial infection by an N-acyl homoserine lactone. Nature. 2001;411:813–7.
39. Bjarnsholt T, Givskov M. Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens. Philos Trans R Soc Lond B Biol Sci. 2007;362:2123–22.
40. Chan KG, Atkinson S, Mathe K, Sam CK, Chhabra SR, Câmara M, Koh CL, Williams P. Characterization of N-acylhomoserine lactone degrading bacteria associated with the Zingeribe officinale (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in Acinetobacter and Burkholderia. BMC Microbiol. 2011;11:51. doi:10.1186/1471-2180-11-51.
41. Wright II JS, Jin R, Novick RP. Transient interference with staphylococcal quorum sensing blocks abscess formation. Proc Natl Acad Sci U S A. 2005;102:1691–6.
42. Janssens JC, Steenackers H, Metzger K, Daniels R, Ptracek D, Verhoeven T, Hermans K, Vanderleyden J, De Vos D, De Kempaerc S. Interference with the quorum sensing systems of Salmonella enterica serovar typhimurium: possibilities and implications. Commun Agric Appl Biol Sci. 2007;72:35–9.
43. Cataldi TRI, Bianco G, Palazzo L, Quaranta V. Occurrence of N-acyl-L-homoserine lactones in extracts of some Gram-negative bacteria evaluated by gas chromatography-mass-spectrometry. Anal Biochem. 2007;361:226–35.
44. Charlton TS, de Nys R, Netting A, Kumar N, Hentzer M, Givskov M, Klejberg S. A novel and sensitive method for the quantification of N-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. Environ Microbiol. 2000;2:530–41.
45. Gould TA, Herman J, Krank J, Murphy RC, Churchill MEA. Specificity of acyl-homoserine lactone syntheses examined by mass spectrometry. J Bacteriol. 2006;188:773–83.
46. Yang YH, Lee TH, Kim JH, Kim EJ, Joo HS, Lee CS, Kim BG. High-throughput detection method of quorum-sensing molecules by colorimetry and its applications. Anal Biochem. 2006;356:297–9.
47. Truelfe H, Palma P, Famiglii G, Cappiello A. An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrom Rev. 2011;30:491–509.
48. Chambers CE, Visser MB, Schwab U, Sokol PA. Identification of N-acylhomoserine lactones in mucopurulent respiratory secretions from cystic fibrosis patients. FEMS Microbiol Lett. 2005;244:297–304.
49. Favre-Bonté S, Pache JC, Robert J, Blanc D, Pechère JC, van Delden C. Detection of Pseudomonas aeruginosa cell-to-cell signals in lung tissue of cystic fibrosis patients. Microb Pathog. 2002;32:143–7.
50. Miller JN, Miller JC. Statistics and Chemometrics for Analytical Chemistry. 4th ed. Harlow: Prentice; 2000.
51. European Commission. Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off J. 2002;221:8–36.
52. Quinn PI, Carter ME, Markey BK, Carter GR. Clinical Veterinary Microbiology. London: Mosby Wolfe; 1994.
53. Cole LK. Otoscopic evaluation of the ear canal. Vet Clin North Am Small Anim Pract. 2004;34:397–410.
54. Nuttall T, Cole LK. Evidence-based veterinary dermatology: a systematic review of interventions for treatment of Pseudomonus otitis in dogs. Vet Dermatol. 2007;18:69–77.
55. Harper WE, Eips JA. Effect of chlorhexidine/EDTA/Tris against bacterial isolates from clinical specimens. Microbios. 1987;51:107–12.
56. Ghibaudi G, Cornegliani L, Martino P. Evaluation of the in vivo effects of Tris-EDTA and chlorhexidine digluconate 0.15% solution in chronic bacterial otitis externa: 11 cases. Vet Dermatol. 2004;15 Suppl 1:65.
57. Guardabassi L, Ghibaudi G, Damborg P. In vitro antimicrobial activity of a commercial ear antiseptic containing chlorhexidine and Tris-EDTA. Vet Dermatol. 2009;21:282–8.
58. Hosseini J, Zdowc I, Golob M, Blagus R, Kular D, Vengu D, Kotnik T. Effect of treatment with Tris-EDTA/chlorhexidine topical solution on canine Pseudomonas aeruginosa otitis externa with or without concomitant treatment with oral fluoroquinolones. Slow Vet Res. 2012;49:133–40.
59. Holden MTG, Chhabra SR, de Nys R, Stead P, Bainton NJ, Hill PJ, Manefield M, Kumar N, Labatée M, England D, Rice S, Givskov M, Salmond GPC, Stewart GSAB, Bycroft BW, Kjelleberg S, Williams P. Quorum-sensing cross talk: interaction and chemical characterization of cyclic dipeptides from Pseudomonas aeruginosa and other Gram-negative bacteria. Mol Microbiol. 1999;35:1264–66.
60. Morgenstein RM, Sostek B, Rather PN. Regulation of gene expression during swarmer cell differentiation in Proteus mirabilis. FEMS Microbiol Rev. 2010;34:753–63.
61. Stankowska D, Czerwonka G, Rozańska S, Gromicka M, Dzadzek J, Kaca W. Influence of quorum sensing signal molecules on biofilm formation in Proteus mirabilis O18. Folia Microbiol. 2012;57:53–60.
62. Tron EA, Wilkie HL, Petermann SR, Rust L. Pseudomonas aeruginosa from canine otitis externa exhibit a quorum sensing deficiency. Vet Microbiol. 2004;99:121–9.
63. Smith EE, Buckley DG, Wu Z, Saengphimphachak C, Hoffman LR, D’Argenio DA, Miller SJ, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A. 2006;103:8987–92.
64. Dunn GM, Brickman TJ, Dworkin MJ. Multicellular behaviour in bacteria: communication, cooperation, competition and cheating. BioEssays. 2008;30:206–8.
65. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature. 2000;407:762–4.
66. Pearson JP, van Delden C, Iglewski BH. Active efflux and diffusion are involved in transport of Pseudomonas aeruginosa cell-to-cell signals. J Bacteriol. 1999;181:1203–10.
67. Nilsson P, Olofsson A, Fagerlin M, Fagerstrom T, Rice S, Kjelleberg S. Active efflux and diffusion are involved in transport of Pseudomonas aeruginosa cell-to-cell signals. J Bacteriol. 1999;181:1203–10.
68. Pearson JP, van Delden C, Iglewski BH. Active efflux and diffusion are involved in transport of Pseudomonas aeruginosa cell-to-cell signals. J Bacteriol. 1999;181:1203–10.
69. Uroz S, Desaux Y, Oger P. Quorum sensing and quorum quenching: the yin and yang of bacterial communication. Chem Bio Chem. 2009;10:205–16.
70. Romero M, Acuña L, Otero A. Patents on quorum quenching: interfering with bacterial communication as a strategy to fight infections. Recent Pat Biotechnol. 2012;6:2–12.
71. Kalia VC. Quorum sensing inhibitors: an overview. Bioessays. 2013;35:224–45.
72. Uroz S, Desaux Y, Oger P. Quorum sensing and quorum quenching: the yin and yang of bacterial communication. Chem Bio Chem. 2009;10:205–16.
73. Romero M, Acuña L, Otero A. Patents on quorum quenching: interfering with bacterial communication as a strategy to fight infections. Recent Pat Biotechnol. 2012;6:2–12.
74. Truchado P, Larrosa M, Ibañez AI, Allende A. Plant food extracts and phytochemicals: their role as quorum sensing inhibitors. Trends Food Sci Tech. 2015;43:189–204.