**Title: Reservoirs of resistance: polymyxin resistance in veterinary-associated companion animal isolates of *Pseudomonas aeruginosa.***

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**Abstract:**

*Pseudomonas aeruginosa* is an opportunistic pathogen and a major cause of infections. Widespread resistance in human infections are increasing the use of last resort antimicrobials such as polymyxins. However, these have been used for decades in veterinary medicine. Companion animals are an understudied source of antimicrobial resistant *P. aeruginosa* isolates. This study evaluated the susceptibility of *P. aeruginosa* veterinary isolates to polymyxins to determine whether the veterinary niche represents a potential reservoir of resistance genes for pathogenic bacteria in both animals and humans.

Clinical *P. aeruginosa* isolates (n = 24) from UK companion animals were compared for antimicrobial susceptibility to a panel of human-associated isolates (n = 37). Minimum inhibitory concentration (MIC) values for polymyxin B and colistin in the companion animals was significantly higher than in human isolates (p=0.033 and p=0.013 respectively). Genotyping revealed that the veterinary isolates were spread throughout the *P. aeruginosa* population, with shared array types from human infections such as keratitis and respiratory infections, suggesting the potential for zoonotic transmission. Whole genome sequencing revealed mutations in genes associated with polymyxin resistance and other antimicrobial resistance-related genes.

The high levels of resistance to polymyxin shown here, along with genetic similarities between some human and animal isolates, together suggest a need for sustained surveillance of this veterinary niche as a potential reservoir for resistant, clinically-relevant bacteria in both animals and humans.

**Introduction**

Antimicrobial resistance (AMR) is of great concern in both human and veterinary healthcare. This issue is confounded by the lack of novel antibacterials being developed in the past 30 years, in particular those with activity against serious Gram-negative infections[1](#_ENREF_1), [2](#_ENREF_2),[3](#_ENREF_3). Therefore older, previously less favoured drugs, such as polymyxins, are increasingly being used [4-8](#_ENREF_4). Polymyxin B and especially polymyxin E (colistin) are used in human and veterinary clinical practice, both systemically and topically, due to their bactericidal activity against a wide range of Gram-negative bacilli including major agents of nosocomial infections such as *Acinetobacter baumannii*[*9*](#_ENREF_9)*, carbapenem-resistant Enterobacteriaceae*[*10*](#_ENREF_10) and *Pseudomonas aeruginosa*[*11*](#_ENREF_11). Polymyxins selectively bind to the lipopolysaccharide (LPS) of the outer cell membrane, disturbing its permeability and causing destabilization and membrane lysis[12](#_ENREF_12). Polymyxin use has been at relatively low levels in human medicine due to safety concerns, in particular nephrotoxicity and neurotoxicity [13](#_ENREF_13). However, colistin has been re-introduced in clinical practice for treating pathogens resistant to all available antimicrobials and is therefore often regarded as a ‘last resort’ treatment in patients with cystic fibrosis (CF) and in nosocomial infections involving *P. aeruginosa* [14](#_ENREF_14).

*P. aeruginosa* is an opportunistic pathogen of both humans and companion animals. In humans, it can cause severe hospital-acquired infections such as pneumonia, keratitis, burn and wound infections, urinary tract infections, endocarditis and meningitis [15](#_ENREF_15), [16](#_ENREF_16). *P. aeruginosa* can affect the lower respiratory system in humans and is an important pathogen in patients with CF and also other chronic lung diseases such as non-CF bronchiectasis [17](#_ENREF_17). In companion animals, *P. aeruginosa* can cause pyoderma, chronic otitis externa, ulcerative keratitis, wound infections, respiratory tract infections and urinary tract infections in a number of species [18-22](#_ENREF_18). A number of other diseases involving *P. aeruginosa* in animals are known, including equine endometritis [23](#_ENREF_23) and in chronic equine wounds where the ability of *P. aeruginosa* to form and survive within protective biofilms aids persistence [24](#_ENREF_24), [25](#_ENREF_25).

Polymyxin B is a common first line topical therapy to treat otitis externa in dogs and cats, and has been used for systemic treatment of endotoxaemia-associated with severe colic and other gastrointestinal diseases in horses [26](#_ENREF_26), [27](#_ENREF_27). High levels of *P. aeruginosa* have been identified in pet and laboratory chinchillas [28](#_ENREF_28). A European Medicines Agency report on colistin usage in animals within the EU stated that across 30 EU/EEA European countries for which sales data are available for 2015, polymyxins were the 5th most sold group of antimicrobials (6.8%), after tetracyclines (32.8%), penicillins (25.0%), sulphonamides (11.8 %), and macrolides (7.2 %)[29](#_ENREF_29). The use of polymyxins specifically in companion animals is not reported in the literature. However, there are several studies looking at antimicrobial prescribing in small animal practice [30-32](#_ENREF_30).

Resistance mechanisms in *P. aeruginosa* to polymyxins include adaptive and intrinsic resistance, often through genetic mutations [33](#_ENREF_33), and are generally characterized by alterations in the outer membrane [34](#_ENREF_34). These mechanisms have recently been reviewed by Jeannot et al[35](#_ENREF_35). A study by Fernandez *et al.* reported a relatively small polymyxin B resistome involving 17 susceptibility/intrinsic resistance determinants, including evidence of cross resistance between colistin and polymyxin B [36](#_ENREF_36). In addition, previous studies have shown that selective pressure generated by the increased use of colistin can lead to emergence of colistin resistance [37](#_ENREF_37), [38](#_ENREF_38). This raises concern regarding use of polymyxins within the veterinary community whereby their use may help drive development of polymyxin B/colistin resistant *P. aeruginosa* strains [39](#_ENREF_39). Such strains could then act as a source of resistant *P. aeruginosa* populations. The occurrence and risk of *P. aeruginosa* transmission between animals and humans is unknown. While it is possible that animals may act as sources of bacteria for humans, transmission is likely to occur both ways. Transmission of *P. aeruginosa* between a CF patient and its pet cat has previously been reported [40](#_ENREF_40).

In this study, we investigated resistance prevalence to polymyxins in veterinary-associated companion animal (including dog, cat and horse) *P. aeruginosa* isolates, and compared their phenotype and genotype to those from a reference panel of human isolates. This provides an important insight into *P. aeruginosa* AMR in this niche, and suggests that potential resistance in animals is a critically important area for future consideration [41](#_ENREF_41), [42](#_ENREF_42).

**Materials and Methods**

**Bacterial isolates**

Clinical *P. aeruginosa* (n = 24) isolates from 22 companion animals attending the Small Animal or the Philip Leverhulme Equine Hospital (both referral hospitals; University of Liverpool, UK) in 2012 were collected for this study as part of routine microbiological diagnostic work up (Table 1). Human-associated *P. aeruginosa* isolates (n=37, [25 CF and 12 non-CF clinical isolates]) from a previously published panel were used for comparison [43](#_ENREF_43), [44](#_ENREF_44). All isolates were stored in Luria-Bertani Broth (LB) (Oxoid) supplemented with glycerol and stored at -80 oC. When needed, bacteria were grown on Columbia agar (Oxoid) aerobically for 24hrs at 37oC.

**Minimum inhibitory concentration (MIC) assays (polymyxin B and colistin)**

For all isolates, MIC for polymyxin B and colistin (Sigma, UK) were performed from overnight broths using EUCAST standardised methods[45](#_ENREF_45). In brief, broth dilutions were performed in 96-well plates with antibiotic concentrations from 128 mg/L to 0.25 mg/L. Positive and negative controls were used throughout and 5 replicate assays were performed. Controls strains *P. aeruginosa* ATCC 27853, the laboratory reference strain PAO1 and *Escherichia coli* NCTC 13846 were used in every experiment. The plates were incubated aerobically at 37oC and assessed after 24 and 48hrs of growth in room air.

For colistin, standard breakpoints were used (>2 mg/L), consistent with the EUCAST guidelines used by human clinical microbiology diagnostic laboratories in the UK[45](#_ENREF_45). MIC values for polymyxin B were interpreted at breakpoints of Sensitive ≤ 2mg/L, Intermediate=4mg/L, Resistant ≥8mg/L (M100-ED29 Clinical and Laboratory Standards Institute)[46](#_ENREF_46).

**Further antimicrobial susceptibility testing**

To test susceptibility to a panel of veterinary-relevant antimicrobials, subcultures were made of the human and companion animal samples by passage on blood agar or Columbia agar. After aerobic incubation at 37oC overnight, colonies were suspended in 5mL sterile diluted water (SDW) to produce a uniform turbidity of 0.5 McFarland units. A 10μL aliquot of this solution was transferred to 11mL cation-adjusted Mueller–Hinton broth (Sigma, UK) that was then transferred to the Sensititre COMPAN1F 96well microtitre plate via the Thermoscientific Sensititre® AIM™ (ThermoScientific ) automated inoculation delivery system. This system determines resistance to ampicillin, amoxicillin/clavulanic acid, ticarcillin, trimethoprim/sulfamethoxazole, gentamicin, penicillin, ceftiofur, enrofloxacin, cefovecin, amikacin, cefpodoxime, imipenem, erythromycin, marbofloxacin, oxacillin, cefoxitin, ticarcillin/clavulanic acid, clindamycin, doxycycline, chloramphenicol, cefazolin and rifampin. The plates were incubated aerobically at 37oC for 24hrs and the plates read using the Sensititre OptiRead™ Automated Fluorometric Plate Reading SystemTrek Sensititre® (ThermoFisher Scientific) to measure the MIC for inhibition of growth (breakpoints provided by Thermo Scientific SENSITITRE COMPAN1F protocol).

For antimicrobials commonly associated with human use, disk diffusion assays were performed to determine antimicrobial susceptibilities for ceftazidime (30 µg), ciprofloxacin (5 µg), meropenem (10 µg), tazobactam/piperacillin (85 µg) and tobramycin (10 µg) (all from Oxoid, Basingstoke, UK) using current EUCAST guidelines[45](#_ENREF_45).

**Alere array tube genotyping for *P. aeruginosa***

Alere Array Tubes (Alere Technologies) were used to genotype the isolates as described previously [47](#_ENREF_47). In brief, a sweep of each isolate was emulsified in 1ml of SDW and centrifuged at 10000g for 2 min. The supernatant was removed and resuspended in 200 µl of SDW, boiled for 5 min and centrifuged at 10000 x g for 2mins. 5 µl of this supernatant was added to 5 µl of a labelling master mix and amplified by PCR (5 min at 96 for 1cycle, 50 cycles of 20 s at 62oC, 40 s at 72 oC and 60 s at 96 oC). Following hybridization and washing, reagent C3 (containing Horse Radish Peroxidase conjugate) was used to label the chip. Detection was performed using the Iconoscan, Iconoclust Software (Alere Technologies). Tube Array images were transformed into array types as previously described [47](#_ENREF_47). In order to study the veterinary isolates in the context of the wider population, an array type database of >900 recorded *P. aeruginosa* strains was used [48](#_ENREF_48). For displaying the wider *P. aeruginosa* population, the eBURST algorithm was applied [49](#_ENREF_49). The number of isolates = 981, number of sequence types = 256, number of loci per isolate =16 and number of resampling for bootstrapping = 1000 (for statistical confidences).

**Statistical analysis:**

SigmaPlot (version 13.0) was used to compare the median MIC values (of 5 replicates) for each isolate. The MIC results for each of the polymyxins (ie. colistin vs polymyxin B) and for each set of isolates (ie. companion animal vs human) were compared. To assess resistance to polymyxin between the human and veterinary isolates Fisher’s Exact Test (with p<0.05 considered significant) was used.

**Whole genome sequencing of bacterial isolates:**

Genomic DNA from the seven companion animal-associated polymyxin resistant isolates was extracted from overnight cultures using the DNeasy Blood and Tissue Kit (QIAGEN). Genomic DNA (500 ng) was mechanically fragmented for 40 sec using a Covaris M220 (Covaris, Woburn MA, USA) with default settings. Fragmented DNA was transferred to PCR tubes and library synthesis was performed with the Kapa Hyperprep kit (Kapa biosystems, Wilmington MA, USA) according to manufacturer’s instructions. TruSeq HT adapters (Illumina, SanDiego, CA, USA) were used to barcode the samples and libraries were sequenced along with 41 other bacterial genomes in an Illumina MiSeq 300 bp paired-end run at the Plateforme d’Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (Laval University, Quebec, Canada)[50](#_ENREF_50). Resistant isolate genomes were assembled using the A5 assembler version A5-miseq 20140521 [51](#_ENREF_51) and annotated using prokka version 1.5[52](#_ENREF_52). Accession numbers are available in Table S1.

All seven polymyxin resistant genomes were aligned to reference genome PAO1 and separately, PA14 using bwa mem version 0.7.5a[53](#_ENREF_53). Resulting .sam alignment files were sorted, converted to .bam format and duplicates marked and removed using picardtools version 1.85. The Genome Analysis Toolkit (GATK) version 3.3 [53](#_ENREF_53) was used to create indel targets, realign them and call variants using the Unified Genotyper(UG) module. All variants were filtered using vcffilter version[54](#_ENREF_54) ‘DP >9’ and ‘QUAL >10’ to produce the final .vcf files. For all isolates, the estimated genome coverage was over x15.

Reference genomes PAO1, PA14, PA7 and LESB58 and 338, which were deemed to represent the wider population[55](#_ENREF_55), were downloaded from the *Pseudomonas* Genome Database [56](#_ENREF_56). The wider population was a random subset of isolates from a previous published study that included isolates from North America, South America, Europe and Asia[55](#_ENREF_55). The core genome was defined and extracted by Panseq[57](#_ENREF_57) as 500bp fragments matching in all 342 genomes with >=85% similarity. MEGA6 [58](#_ENREF_58) was used for all phylogenetic analyses. Phylogeny was estimated using the maximum likelihood method (ML) and Tamura-Nei substitution model from concatenated polymorphic sites within the defined core genome. Inner node support was based on 100 bootstrap replicates. The tree was drawn using Figtree[59](#_ENREF_59).

Ortholog sequences for 31 polymyxin resistance-associated genes were downloaded from the Pseudomonas Database[56](#_ENREF_56).  A custom blast database was curated consisting of the seven polymyxin resistant isolate genomes using the BLAST+[60](#_ENREF_60) makeblastdb software. Each polymyxin resistance-associated gene was aligned against the custom database using BLAST+ blastall. A python script was used to extract matching regions and convert to amino acid sequences from the seven polymyxin resistant isolate genomes.

**Results**

**Resistance to polymyxin antimicrobials**

For the veterinary isolates, 92% displayed resistance to polymyxin B (Table 2). The highest MIC value was observed in an isolate (984) detected from a canine ear, which had an MIC of >128 mg/L (Figure 1). In comparison, 68% of the human-associated *P. aeruginosa* isolates were resistant to polymyxin B (p=0.033). The proportion resistant to colistin amongst the veterinary isolates was also significantly higher compared to the human-associated panel (54% compared to only 22%, p=0.013) (Table 2). The number of colistin resistant isolates was surprisingly high however, comparison to previous data[43](#_ENREF_43) is challenging as the MIC cut-off value has recently changed from >4 mg/L to >2 mg/L[61](#_ENREF_61). Many of the human isolates displayed MICs of around 4 mg/L and 2 mg/L and therefore caution must be taken when interpreting these results. The isolates with the highest MIC value to colistin were isolates 856 and 903 (Figure 1). These isolates were both of equine origin, from an abdominal incision and a urine sample respectively. Minimum inhibitory concentration (MIC) 50 values for polymyxin B in the companion animal and human isolates were 16 mg/L and 8 mg/L, respectively, while the MIC50 values for colistin were 4 mg/L and 2 mg/L respectively (Table 2).

**Resistance of veterinary-associated *P. aeruginosa* to other antimicrobials**

Testing of a panel of antimicrobials included in the Sensititre COMPAN1F microtitre plates (Table 3) showed that there was no resistance to amikacin amongst the companion animal isolates and only intermediate resistance to imipenem. Four percent of isolates were resistant to gentamicin and ticarcillin/clavulanic acid. Higher levels of resistance were detected to ticarcillin (21%), marbofloxacin (21%) and enrofloxacin (33%). As expected, high resistance to ceftiofur was detected with 92% of isolates classed as resistant (Table 3).

The human isolates were also tested using the Sensititre COMPAN1F microtitre susceptibility assay. A similar trend in resistance was observed with the highest number of isolates showed resistance to ceftiofur and the lowest to amikacin (Table 3).

Based on disc diffusion susceptibility assays, no resistance was detected to ceftazidime, meropenem or tobramycin (data not shown). Two isolates were resistant to ciprofloxacin and a further three displayed intermediate resistance. The two resistant isolates (1055 and 823) were both of canine origin (Figure S1). The isolates with intermediate resistance were from a canine buccal swab (1095) and the left and right ear (467L and R) of the same dog.

**Circulating strain types of *P. aeruginosa***

The Alere Array Tube has been used to determine the population structure of *P. aeruginosa* [47](#_ENREF_47) and a database of 955 isolates can be used to infer the wider population [48](#_ENREF_48). This typing technique was used here for the first time to classify the companion animal isolates. From the 24 isolates, 20 different array types were identified (Table 1). Eight were novel array types not previously identified in the database [48](#_ENREF_48). One array type had previously been associated only with the environment (isolate 2C12 from water). However, the remaining array types had been previously associated with human infections such as keratitis, catheter-associated and respiratory tract infection in CF and pneumonia (Table 1). Using the database of array types to generate a *P. aeruginosa* population structure, the isolates were mostly distributed amongst the main *P. aeruginosa* clades. Five isolates were located as outliers (isolates 811, 1107, 856) and the two isolates from the same dog (467L and 467R) (Figure 2). The latter two isolates share an array type with the widely used laboratory strain of *P. aeruginosa*, PAO1.

**Genome sequencing and analysis of the polymyxin resistome**

Seven veterinary-associated isolates that displayed resistance to colistin and polymyxin B were selected for whole genome sequencing. A phylogenetic tree of the veterinary isolates and 342 of the available *P. aeruginosa* available genomes (Figure 3) shows that four of the isolates were located in clade I and another two in clade II of the main *P. aeruginosa* population. Although none of the isolates were found to be PA7-like, isolate 856 (equine origin) was also diverse from the main population, located on a new arm of the phylogenetic tree (Figure 3). This genome had 125303 SNPs and 1884 indels compared to PAO1 and 130312 SNPs and 1985 indels in comparison to PA14 (Table S2). This was double the number of SNPs and indels observed for other sequenced isolates.

Thirty-one genes associated with resistance to polymyxins were analysed (Table S3). Multiple stop codons were identified in isolate 856. A stop codon in *pyrC* was identified in isolate 1098. Additional amino acid modifications resulting in a change in hydrophobicity were identified in all seven genomes (Table 4).

In addition, the genome sequences were analysed using the Comprehensive Antibiotic Resistance Database (CARD) database [62](#_ENREF_62) (Figure S2). This showed the presence of resistance determinants including beta-lactamase (PDC-1-7, *amr*), efflux (*smeB, mexBDFIY*), fluoroquinolone and elfamycin resistance. Despite being the most divergent isolate by sequencing, isolate 856 had the fewest additional resistance genes.

**Discussion**

In this study, we describe a higher prevalence of resistance to the polymyxin antimicrobials polymyxin B and colistin in a panel of *P. aeruginosa* isolates from companion animals from the UK compared to human-associated isolates[43](#_ENREF_43). To our knowledge, this is the first description of increased resistance to colistin amongst companion animal clinical isolates. Using molecular typing methods, we identified that some of the strain types from animals have previously been identified in human infections. Genome sequencing revealed mutations leading to changes in the amino acid sequences that could contribute to resistance and revealed the highly diverse nature of some of the isolates. No resistance to some commonly used human antimicrobials (including meropenem, ceftazidime and tobramycin) was found; however five isolates showed either resistance or intermediate resistance to the high priority critically important antimicrobials fluroquinolone (ciprofloxacin). For the majority of the human clinically-relevant antimicrobials there was little evidence for cross-resistance with veterinary antimicrobials however there did appear to be potential cross-resistance between the fluoroquinolones enrofloxacin and ciprofloxacin.

Several isolates within our companion animal sample set were from dog skin (including wounds) or ears. In canids, *P. aeruginosa* is a frequently isolated pathogen in chronic otitis externa and otitis media [22](#_ENREF_22). The recommended management of canine otitis externa consists of identifying and treating the predisposing factors and primary disease, ear cleaning/flushing, appropriate topical therapy and if indicated, systemic antimicrobial medications [63](#_ENREF_63). First line topical therapies for canine otitis externa commonly contain polymyxin B as one of the active ingredients. It is authorised for use in several ear conditions and skin infections. A recent report examining antimicrobial susceptibility profiles of bacterial isolates in the canine ear in Australia identified *P. aeruginosa* as one of the five most commonly isolated bacterial pathogens [64](#_ENREF_64). Of 3541 canine ear swabs, 35.5% isolated *P. aeruginosa*. However, although they raised concerns of resistance levels in the other Gram-negative bacterial isolates (*Escherichia coli* and *Proteus* spp*.*) to polymyxin B, the resistance in *P. aeruginosa* isolates was comparatively low to polymyxin B (7%) and gentamicin (5%)[64](#_ENREF_64). This is in contrast to the findings presented in our study whereby polymyxin resistance levels of veterinary isolates were high. The precise reason for such differences is uncertain but may represent local differences in antimicrobial usage patterns and that the centres involved in this study were specialist referral centres and therefore potentially bias towards infections that display greater antimicrobial resistance.

As well as this use for canine otitis externa, Polymyxin B, in the form of polymyxin B sulphate administered intravenously, is reported for use in equids as a treatment of endotoxaemia[65](#_ENREF_65). Colistin is not used therapeutically in companion animals in the UK although it is available as an authorised product for administration in various food production animals for the indication of enteric infections. However, there is little evidence in this study as to what the drivers of resistance may be within the companion animal niche of *P. aeruginosa* isolates.

Resistance to polymyxins has been attributed to modifications to the outer membrane including modifications to lipid A and LPS as well as two-component regulators. A study by Fernandez et al. reported a relatively small polymyxin B resistome involving 17 susceptibility/intrinsic resistance determinants [36](#_ENREF_36). They also demonstrated considerable cross-resistance in susceptibility to polymyxin B and to colistin [36](#_ENREF_36), suggesting resistance determinants confer resistance to both antimicrobials. However, other genes have also been implicated in resistance [66](#_ENREF_66). Seven of the veterinary isolates, chosen for their extreme resistance, were whole genome sequenced and mutations, surprisingly this included stop codons (a severe mutation resulting in early termination of transcription and therefore no or highly altered protein production), that were detected in genes previously implicated in polymyxin resistance such as *pyrC, wapR, mpl* and *ampR.* Many of the sequences studied showed modifications in genes leading to an altered amino acid, several of which had altered hydrophobic/hydrophilic properties which can result in conformational or activity changes in the protein. It is possible that a combination of these mutations may lead to a change in phenotype through membrane remodelling [66](#_ENREF_66) although further studies would be needed to confirm this.

Using the array tube typing method, the *P. aeruginosa* veterinary isolates were found to be distributed throughout the *P. aeruginosa* population. These findings highlight the potential of transmission of these bacteria between humans and animals. Using whole genome sequencing on a limited subset of isolates, the veterinary isolates were generally clustered within the main *P. aeruginosa* population; however one isolate, 856, did not cluster with any other previously sequenced isolate. Through mapping to the genomes of both PAO1 and PA14, we found that this isolate had double the number of SNPs and indels shown by any of the other isolates sequenced. The isolate was of equine origin and highlights the importance of studying alternative and underrepresented niches in order to fully characterise the *P. aeruginosa* pan genome.

In conclusion, these findings raise concerns regarding the use of polymyxins within the veterinary community. Such isolates (from both dogs and horses) could then potentially act as a source of resistant isolates for the human-associated *P. aeruginosa* population. The findings suggest that future surveillance of polymyxin resistance in animal-associated *P. aeruginosa* strains is warranted. In this study, there was a small sample size from only one geographical location and no detailed history of prior antibiotic usage. These limitations warrant further, much larger studies. The veterinary setting is an often ignored niche that provides close proximity between humans and companion animals and therefore cross infection of resistant organisms would be possible. The preservation of colistin as a “last resort” effective anti-pseudomonal drug is of great concern.

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Figures and Tables.

Figure 1. Resistance of *P. aeruginosa* to polymyxins in isolates from companion animals and humans. Polymyxin B resistance in A). veterinary isolates and B) human isolates. Colistin resistance in C). veterinary isolates and D) human isolates.

Figure 2. Distribution of the array tube codes of the veterinary *P. aeruginosa* isolates amongst a larger panel of *P. aeruginosa* isolates using eBurst analysis. Veterinary isolates highlighted in red.

Figure 3. Maximum likelihood tree using the Tamura-Nei substitution model of *P. aeruginosa* core genome SNPs. The core genome is defined as polymorphic sites within 500bp fragments matching => 85% similarity in 342 genomes. The tree is based on 1336 phylogenetically informative polymorphic sites after gaps and N's were removed. Three clear groups are distinguishable: that containing PA14, PAO1 and PA7 respectively. Branches to PA7 and 856 have been truncated for clarity, so an impression of the real distances in the same tree has also been provided.

**Table 1** Companion animal bacterial isolates used in this study including Alere Array Tube Hexidecimal codes of veterinary *P. aeruginosa* isolates. CF-cystic fibrosis, COPD-chronic obstructive pulmonary disorder, ICU-intensive care unit.

|  |  |  |  |
| --- | --- | --- | --- |
| Strain or Isolate | Description | Array Tube Code | Additional details on isolates with same array tube code (Cramer et al., ) |
| Animal strains |  |  |  |
| 1115 | Canine testicle/ scrotum swab | **AF92** | COPD, USA. |
| 1114 | Canine neck wound swab | **2C12** | Water, Germany |
| 1107 | Canine Bile sample | **2422** | Novel |
| 1098 | Canine Vaginal swab | **B429** | Keratitis, UK.  River, Germany. |
| 1095 | Canine Buccal swab | **AA0A** | Novel |
| 1090 | Canine Skin lesion swab | **2O22** | Novel |
| 1070 | Canine neck swab) | **2D92** | Novel |
| 1055 | Canine Lip fold swab | **0812** | Found in CF patients, bacteraemia and ICU, France, Switzerland and Austria. |
| 994 | Canine Right elbow swab | **0C12** | CF sputum Germany. |
| 984 | Canine Right ear swab | **241A** | Acute infection, source unknown.  Catheter, Germany. |
| 982 | Canine Urine sample | **2FAA** | CF throat swab, Water, Italy and Germany |
| 978 | Canine Non-Healing Wound swab | **6C12** | CF, Italy. COPD, USA |
| 970 | Canine Wound swab | **0B9A** | Novel |
| 969 | Canine Oropharyngeal tube swab | **840A** | Keratitis, Bristol. |
| 897 | Canine ear swab | **848A** | Novel |
| 886-1 | Canine nail pus swab | **2B92** | Novel |
| 992 | Feline Oropharyngeal tube stoma swab | **AF92** | COPD, USA. |
| 856 | Equine Abdominal incision swab | **B420** | Sputum, nose and water, Germany. |
| 903 | Equine Urine sample | **B429** | Keratitis, UK and River Germany. |
| 823 | Canine Left ear swab | **241A** | Acute infection, source unknown.  Catheter, Germany. |
| 811 | Equine nasal swab | **AA01** | Novel |
| 467-L | Canine Left Ear swab | **0002** | PAO1 group. CF Germany.  Pneumonia, Switzerland. |
| 467-R | Canine Right Ear swab | **0002** | PAO1 group. CF Germany.  Pneumonia, Switzerland. |
| 886-2 | Canine nail pus swab | **2B92** | Novel |

**Table 2.** Minimum inhibitory concentration tests to show antimicrobial resistance of *P. aeruginosa* in companion animal (n=24) and human (n=37) isolates to polymyxin antimicrobials

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Isolate | Antimicrobial | Resistance Breakpoint  (mg/L) | Resistant Strains  (n) | Resistant Strains  (%) | MIC50  (mg/L) | MIC90  (mg/L) |
| Animal | Polymyxin B | ≥8 | 22 | 92 | 16 | 32 |
| Animal | Colistin | >2 | 13 | 54 | 4 | 8 |
| Human | Polymyxin B | ≥8 | 25 | 68 | 8 | 16 |
| Human | Colistin | >2 | 8 | 22 | 2 | 4 |

**Table 3.** Antimicrobial resistance of *P. aeruginosa* isolated from companion animals (n=24) and human isolates (n=37)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Antimicrobial | | Breakpoint mg/L | Resistant strains (*n)*  An Hu | Intermediate strains (*n*)  An Hu | Susceptible strains (*n*)  An Hu | Resistant strains (%)  An Hu |
| Amikacin | S=8, I=16, R>16 | | 0 2 | 0 3 | 24 32 | 0 [0,0] 5.4 [1.5,18] |
| Ceftiofur | S=2, I=4, R>4 | | 22 35 | 2 2 | 0 0 | 92 [74,98] 95 [82,99] |
| Enrofloxacin | S=0.5, I=2, R>2 | | 8 13 | 13 21 | 3 3 | 33 [18,53] 35 [22,51] |
| Gentamicin  Imipenem  Marbofloxacin  Ticarcacillin  Ticarc/clavulanic acid | S=4, I=8, R>8  S=4, I=8, R>8  S=1, I=2, R>2  S=64, R>64  S=64, R>64 | | 1 10  0 8  5 13  5 8  1 7 | 0 0  2 7  2 0  0 0  0 0 | 23 27  22 22  17 24  19 29  23 30 | 4[1,20] 27 [15,43]  0[0,0] 22 [11,37]  21 [9,41] 35[22,51]  21[9,41] 22[11,37]  4 [1,20] 19[10,34] |

**Confidence intervals are 95% [Lower, Upper] Wilson method. An=animal, Hu=human**

Table 4. Amino acid changes (presence of stop codons and changes in hydrophobicity) identified in the seven veterinary *P. aeruginosa* isolates by whole genome sequencing.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| *Function* | Gene | 856 | 886 | 903 | 994 | 982 | 1090 | 1098 |
| *Cell metabolism-associated* | *pyrC* | G423STOP |  |  |  |  |  | G423STOP |
| *pdxB* |  |  | V109K |  | D324A |  | V109K |
| *aroB* | A200E | A200E | A200E | A200E | A200E | A200E | A200E |
| *Acquired/adaptive resistance through LPS modification* | *parR* | L153R |  | L153R | L153R | L153R |  | L153R |
| *parS* | A115E |  |  |  |  |  |  |
| *Recycling of cell wall components* | *mpl* | A404P  E451STOP |  |  |  |  |  |  |
| *Transcriptional regulator-associated with resistance to antibiotics* | *ampR* | E114A  M228R  R291STOP | E114A  E172V | E114A  M228R |  |  |  | E114A  M228R |
| *Activation of LPS-modifying operon by mutations in TCSs* | *pmrA* | L71R | L71R |  | L71R |  | L71R | L71R |
| *LPS biosynthesis-related functions* | *wapR* | W114R  295STOP |  |  |  |  |  |  |
| *Modification of lipid A or Kdo with aminoarabinose* | *arnC* | P23A |  |  |  |  |  |  |
| *arnD* | A284D |  |  |  |  |  |  |
| *arnF* | P137L |  |  |  |  |  |  |

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