Phenotypical Comparison of Pseudomonas Aeruginosa Isolated from Human and Veterinary Samples; Impact of Host Adaptation on Infection Pathogenesis

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Research Article

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Abstract

**Purpose:** Determinants of virulence in *Pseudomonas aeruginosa* vary strongly depending on its habitat. In this study, we analyzed these alterations depending on the host organism in isolates cultured from canine ears and compared it to clinical extended-spectrum antibiotic-resistant *Pseudomonas aeruginosa* isolates (XDR), clinical antibiotic-sensitive (non-XDR) from humans and environmental isolates (EI) analyzed during our first study in 2017.

**Methods:** A total of 22 veterinary isolates cultured from canine ears (VET) were examined for spontaneous biofilm formation, stress response in biofilm formation induced by meropenem, in vitro fitness, susceptibility to human serum and polymorphonuclear leukocytes and the genetically determined virulence factors *toxA*, *exoS*, *exoT*, *exoU*, *exoY*, *nan1*, *cif*, *lasA* and *lasB*.

**Results:** We observed significantly elevated spontaneous biofilm formation and serum susceptibility in VET isolates compared to EI and non-XDR isolates as well as significantly decreased in vitro fitness compared to XDR isolates. The VET isolates resembled most the XDR subgroup of isolates previously cultured from blood. Within the environmental isolates, we observed an increase of spontaneous biofilm formation and *exoU* presence in isolates cultured from community water samples over hospital water samples to pool samples.

**Conclusions:** Considering the distinct differences in some features of the examined VET isolates, a higher degree of phenotypical adaption can be assumed. Increased biofilm formation seems to be a common and characteristic event in isolates adapted to a specific habitat. Therefore amplification of potentially more virulent *Pseudomonas aeruginosa* strains in domestic animals may lead to elevated zoonotic risk for example for pet owners.

Introduction

Among the most relevant facultative pathogenic organisms, *Pseudomonas aeruginosa* (*Pa*) is a Gram negative bacterium that causes nosocomial infections and is one of the most important pathogens for patients with cystic fibrosis. Although *Pa* inhabits a wide spectrum of habitats, including moist environments like in-house water supplies, the genetic similarity between isolates of different origins, including human and veterinary clinical isolates, is surprisingly high [1]; and, in veterinary medicine, equally sporadic diseases and outbreaks occur [2–7]. Due to this wide spectrum of hosts, a zoonotic potential exists [8, 9].

In a recent study, we revealed alterations in pheno- and genotypical properties between clinical extended-spectrum antibiotic-resistant isolates (XDR), non-multiresistant clinical isolates (non-XDR) and environmental isolates cultured from community-based water samples (EI), including significantly higher spontaneous and induced biofilm formation in XDR than in non-XDR clinical isolates. Furthermore, XDR isolates showed increased in vitro fitness but were more susceptible to normal human serum than both
non-XDR and EI. The endemic XDR strains ES-1 and ES-1 showed properties with values at the extremes of the distributions, often with opposite manifestations for particular properties [10].

The present study examines an additional group of 22 isolates cultured from canine ears (VET). This group was chosen because of its importance in veterinary medicine and the possibility of exposure and transmission to animal keepers. Furthermore, we split the already in 2017 evaluated XDR and EI isolates into subgroups, consisting of isolates cultured from similar clinical material to assess their properties in relation to infection site.

*Pa* is primarily considered a cause of nosocomial infections in humans. We wanted to extend the spectrum of our observations by adding a group of clinical isolates from a distinct material of animal origin to learn more about *Pa* in veterinary medicine and to elucidate the mechanisms of persistence and pathogenesis of *Pa*.

**Methods**

Samples consisted of a total of 22 *Pa* isolates of clinical material from canine ears (VET) sampled in various veterinary clinics or practices. These were cultivated and determined in 2015 and 2016 at the Institute for Hygiene and Infectious Diseases of Animals at the University of Giessen.

Of the 296 isolates cultured at Heidelberg University Hospital from 2009 to 2014, 211 clinical extended-spectrum antibiotic-resistant isolates (XDR), which are resistant to piperacillin, ceftazidime, ciprofloxacin and meropenem [11], and 47 environmental isolates cultured from community-based water samples (EI) were subdivided further. The 45 non-multidrug resistant clinical isolates (non-XDR) resistant to 2, at most, of the aforementioned antibiotics have not been subdivided further.

The XDR isolates used in Kaiser et al. (2017) were subgrouped as follows: 15 XDR blood isolates cultured from blood samples, 20 XDR invasive isolates cultured from abscesses and intraoperative swabs, 28 XDR wound isolates cultured from wounds, 55 respiratory isolates cultured from samples of the upper and lower respiratory tract, 31 XDR urinary isolates cultured from urine and urinary tract swabs, 22 XDR colonizing isolates cultured from samples of rectal and vaginal swabs, 15 cfXDR isolates cultured from sputum of cystic fibrosis patients, the endemic strains ES-1 (13 isolates) and ES-2 (12 isolates) cultured from various clinical materials and assigned with RAPD PCR with primers 208 and 272 [12].

The EI isolates cultured from in-house water were subdivided into 10 EI community isolates from community water supply, 25 EI hospital isolates from drinking water samples from hospital facilities and 10 EI pool isolates from pool water samples.

To increase comparability to other studies, the widely used reference strains PAO1 and PA14 were included.

Our methods are described in detail in Kaiser et al. (2017) [10].
To determine **biofilm formation**, test isolates adjusted to the exponential growth phase and a McFarland standard of 0.25 were grown for 18 h in M63 minimal medium in 96-well microtiter plates. Biofilm formation was determined using the optical density of crystal violet bound in the biofilm matrix, by staining the bacterial biofilm with crystal violet and eluting in ethanol. A clinical isolate of the XDR group was used as a positive control and reference isolate. This was done for the biofilm-index\(_{\text{spontaneous}}\) without the addition of antibiotics and for the biofilm-indices\(_{\text{MPMX}}\) with meropenem (MPM) at concentrations of 1 mg/L, 4 mg/L and 125 mg/L. Furthermore, a stress response for each MPM concentration was generated, which was calculated as log10 (biofilm-index\(_{\text{spontaneous}}\)/biofilm-index\(_{\text{MPMX}}\)).

The **in vitro fitness** was determined in a competitive growth assay with a reference isolate. Stationary-phase test and competitor isolates, adjusted to a McFarland index of 0.5, were incubated together for 24 hours at 37°C with constant shaking. The ratio of the two isolates was determined by comparing colony growth on a blood agar plate and an agar plate containing imipenem with the formulae log10(cfu\(_{\text{test isolate}}\)/cfu\(_{\text{competitor}}\)). The reference strain PA14 was chosen for the XDR group, and an XDR isolate with balanced in vitro fitness to PA14 was chosen for the other groups. A high initial bacterial concentration was used to emphasize assertiveness.

**Serum susceptibility** was determined using a killing assay. A suspension with the test isolate, adjusted to a stationary phase and CFU count of 2 X 10^3 cells/ml, and a 10% normal human serum was incubated for 30 min at 37°C, and the CFU reduction was determined and compared to a control sample with 10% heat inactivated normal human serum (30 min; 56°C). CFU counts were detected using the pour plate method on TSA after incubation for 48 h at 37°C.

**PMN susceptibility** was determined using a killing assay under the same conditions, including serum susceptibility with inactivated human serum and a polymorphonuclear neutrophil leukocytes (PMN) concentration of 10,000/ml and compared to the control without PMN using the poor plate method. PMNs were purified from fresh human whole blood taken from healthy volunteers and separated using Polymorphprep (Axis-Shield, Oslo, Norway).

The **genetically determined virulence factors** toxA, exoS, exoT, exoU, exoY, cif, nan1, lasA and lasB were detected by polymerase chain reaction with the following parameters: 94°C for 2 min; 36 cycles (94°C for 30 min; 58°C for 30 min; 68°C for 10); and 68°C for 10 min. Primers were used as described in Atzél et al. (2008), Kaszab et al. (2010) and Kaiser et al. (2017) and are shown in Table 1 [10, 13, 14].

Statistical analysis was performed using the R 3.2.4 software package. For categorical and continuous variables, chi-square tests and Wilcoxon signed-rank tests were used to determine significance, with P values of ≤0.05 indicating statistical significance.

**Results**
Phenotypical properties observed are shown in Table 2. Spontaneous biofilm formation was significantly higher in VET isolates, such as XDR and EI isolates, than in human non-XDR isolates. Within the EI isolates, we observed an increased spontaneous biofilm formation for EI pool isolates compared to EI hospitals and EI community isolates.

The stress response showed both isolates with induced and inhibited biofilm formation within each group at all MPM concentrations. Although generally less pronounced in the XDR isolates, inhibition predominated in all groups and MPM concentrations, with except for the cfXDR isolates at the subinhibitory level of 1 mg/L and the endemic strain ES-2 only at the highest used concentration of 125 mg/L.

A significantly higher in vitro fitness was observed in XDR isolates than in all other groups (varying p-values) except for the cfXDR isolates, which had the second lowest in vitro fitness. The VET isolates showed higher fitness than both EI and non-XDR isolates, with a significant difference in EI isolates (p<0.001).

VET isolates, such as XDR isolates, showed significantly higher serum susceptibilities than EI (p<0.0001) and non-XDR isolates (p<0.01). The cfXDR and XDR blood isolates had particularly high serum susceptibilities, although for these groups, increased exposure to the immune system must be assumed. The serum-susceptible isolates (log10-CFU-reduction by NHS >2.0) also showed significantly higher spontaneous biofilm formation (p<0.01).

In terms of PMN susceptibility, the non-XDR group showed significantly lower values (varying p-values) compared to the other groups.

The genetically determined virulence factors are shown in Table 3. Note that cif, toxA and exoT were present in almost all isolates, and lasA and exoY were present in most of the isolates. The exoS and exoU genes were always exclusive.

Discussion

With regard to some properties showing values at the margins of the distribution, our VET isolates differed distinctly from our EI and non-XDR isolates, which presumably reflecting a generally less adapted and specialized phenotype of Pa. Therefore, a higher degree of phenotypical adaption to certain reservoirs can be assumed.

Increased spontaneous biofilm formation does not seem to be an uncommon adaptation to harsh environmental habitats, such as those of EI pool isolates or isolates adapted to habitats within mammalian hosts, such as our cfXDR isolates or possibly our VET isolates. A connection between adaptation and elevated spontaneous biofilm formation has been shown for Pa [15, 16] and for other species, such as Staphylococcus aureus [17, 18] and Acinetobacter baumannii [19]. In addition, biofilm formation has been shown to be related with both factors associated with persistence and virulence, like
higher resistance to disinfection [20, 21], an essential role in pathogenesis in various infection [22], an association with persistent infections, especially in cystic fibrosis patients [23] and increased mortality in hospitalized patients with bloodstream infections [24].

Because of their different antimicrobial resistance levels, comparing the stress responses of XDR isolates to other isolates is a challenge; however, the wide range of phenotypes within these groups is striking. The diverse manifestations and the characteristic stress response of ES-2 indicate that inducible biofilm formation is presumably much more important in vivo than is represented in the literature and such biofilm formation may demonstrate even wider variations when other stimulants are considered [10, 25–29].

In particular, the capacity to build a high quantitative amount of biofilm is one of the outstanding properties of the two endemic strains and the cfXDR isolates studied herein, which underscore the connection between biofilm formation and persistence. Interestingly, these groups seem to be adapted to different environments, such as lung tissue for the cfXDR group and probably the hospital setting for endemic strains. Groups showing high spontaneous biofilm formation may be related via the pathogenesis of infections with these isolates. Isolates showing high spontaneous biofilm formation in vitro may behave in a similar way in vivo and build biofilms more readily than isolates that show lower in vitro levels of spontaneous biofilm formation.

There may also be a connection between spontaneous biofilm formation and high serum susceptibility in XDR and VET isolates. This is in sharp contrast to what is observed for EI and non-XDR isolates. In clinical isolates with high serum susceptibility - most obviously XDR blood isolates, one of the most susceptible groups to normal human serum and likely the group with the highest exposure - there must be a mechanism to compensate for this disadvantage, namely high biofilm formation. Furthermore, biofilm formation has been connected to reduced motility [15, 30], so a connection with pathogenesis is possible. For these isolates, the route of infection is likely the spread of established biofilm communities or early biofilm manifestation by recently transferred Pa, rather than active colonisation.

Although we observed higher in vitro fitness values for VET isolates compared to EI and non-XDR isolates, the high value obtained for XDR isolates was remarkable. Previous studies have indicated a negative association between inserted antibiotic resistance and in vitro fitness [31–33]. Nevertheless, compensation mechanisms have been demonstrated [32, 34]. Interestingly, in our study, those groups that seem to be phenotypically adapted at higher levels also show higher in vitro fitness values. An important exception are the cfXDR isolates, possibly because this group went through the adaptation process in a habitat with lower microbiological competitive pressure, given that significant geno- and phenotypical alterations have been shown in CF-Isolates [35, 36]. The intermediate in vitro fitness observed in VET isolates may be an early form of this adaption. An important methodical aspect may be that these tests were carried out with a high initial bacterial count, such that the high in vitro fitness observed may be the consequence of assertiveness rather than a high division rate.
The differences in the frequencies of *nan1*, which is associated with respiratory manifestation and adhesion to certain cells [9, 37], and *lasB*, which is associated with cell invasion [38], may also indicate an alteration of the path of infection in XDR isolates. The similarity between the XDR isolates and the water groups, which both had higher *exoU* proportions, is interesting. It appears that *exoU* frequency increases in living environments that are near humans.

The XDR group demonstrated characteristics that are particularly associated with persistence and, as a result, an alteration in virulence properties. In contrast to the non-XDR group, there appears to be a shift in the path of infection in the XDR group towards the passive spread of biofilms. Interestingly, the XDR respiratory isolates, which have particular clinical importance, did not show properties as distinct as those of most of the other XDR subgroups, which may reflect a pathogenesis specific to respiratory infections. This explanation may even apply to XDR wound isolates, which resemble most of the non-XDR group in their properties.

Within EI, we observed an elevation of spontaneous biofilm formation as well as *exoU* frequency in EI pool isolates compared to EI hospitals and EI community isolates. These properties seem to align with our observations for XDR and VET isolates, the closer environments they are obtained from is associated with people. Given the living environment of *Pa* from which we obtained our XDR isolates, adaptation to a human-associated environment seems more likely than direct adaptation to human tissue. One major exception is the cfXDR group, which shows unique properties and is presumably phenotypically adapted directly to human respiratory tissue [35, 39, 40].

In this study, veterinary isolates resembled XDR isolates and particularly XDR blood isolates with regard to many properties, particularly the combination of elevated biofilm formation, high serum susceptibility and genetically determined virulence factors. This may be the result of advanced adaptation in both groups, most likely to an environment close to the mammalian host in regard to the pathogenesis of infection or to the mammalian host itself. Whether this special phenotype is a consequence or a prerequisite for colonisation cannot be answered without further studies. However, it is disturbing that the veterinary isolate resembles the presumably most virulent XDR group obtained from clinical material. Notwithstanding the reasons why veterinary isolates show this pattern of features, amplification of potentially more virulent *Pa* strains, potentially leading to elevated zoonotic risks in immunocompromised and cystic fibrosis patients, must be considered.

**Declarations**

**Ethics approval and consent to participate:**

All used isolates were routinely collected in the microbiology laboratory of the Heidelberg University Hospital or the Institute for Hygiene and Infectious Diseases of Animals of the University of Giessen and stored at -70°C. The current study thus is descriptive of those isolates. Data collected from patients was
anonymized and restricted to possible clinical symptoms of infection. Ethical approval and informed consent statements were therefore not required.

**Consent for publication:**

Not Applicable

**Availability of data and material:**

All data and materials are fully available without restriction. All relevant data are within the manuscript and its Supporting Information files.

**Competing interests:**

The authors have no relevant financial or non-financial interests to disclose.

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**Authors contributions:**

conceptualization: FG, SJK; Methodology: FG, SJK; Formal analysis and investigation: SJK, ADR; Writing - original draft preparation: FG, SJK; Writing - review and editing: FG, SJK; CE; Supervision: FG. Each author participated actively in conducting analyses, drafting sections of the manuscript, editing, and approving the final, submitted version.

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