**Pseudomonas aeruginosa** variants obtained from veterinary clinical samples reveal a role for cyclic di-GMP in biofilm formation and colony morphology

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**Abstract**

Overuse of antibiotics is contributing to an emerging antimicrobial resistance crisis. To better understand how bacteria adapt tolerance and resist antibiotic treatment, *Pseudomonas aeruginosa* isolates obtained from infection sites sampled from companion animals were collected and evaluated for phenotypic differences. Selected pairs of clonal isolates were obtained from individual infection samples and were assessed for antibiotic susceptibility, cyclic di-GMP levels, biofilm production, motility and genetic-relatedness. A total of 18 samples from equine, feline and canine origin were characterized. A sample from canine otitis media produced a phenotypically heterogeneous pair of *P. aeruginosa* isolates, 42121A and 42121B, which during growth on culture medium respectively exhibited hyper dye-binding small colony morphology and wild-type phenotypes. Antibiotic susceptibility to gentamicin and ciprofloxacin also differed between this pair of clonal isolates. Sequence analysis of *gyrA*, a gene known to be involved in ciprofloxacin resistance, indicated that 42121A and 42121B both contained mutations that confer ciprofloxacin resistance, but this did not explain the differences in ciprofloxacin resistance that were observed. Cyclic di-GMP levels also varied between this pair of isolates and were shown to contribute to the observed colony morphology variation and ability to form a biofilm. Our results demonstrate the role of cyclic di-GMP in generating the observed morphological phenotypes that are known to contribute to biofilm-mediated antibiotic tolerance. The generation of phenotypic diversity may go unnoticed during standard diagnostic evaluation, which potentially impacts the therapeutic strategy chosen to treat the corresponding infection and may contribute to the spread of antibiotic resistance.

**INTRODUCTION**

Antimicrobial resistance (AMR) is a serious global threat to medical and veterinary hospitals, resulting in fewer effective treatment options and increased costs for care [1]. Overuse and misuse of antibiotics has led to an increase in the incidence of bacterial infections that are highly resistant to first-line antibiotics [2]. The recent and alarming discovery that some bacteria have acquired resistance to colistin, ‘an antibiotic of last resort’, confirms that the antimicrobial resistance crisis is now a reality and illustrates the current challenges facing clinicians in both human and veterinary medicine [3]. This increase in antibiotic resistance is also coupled with decreasing budgets for research and development of new antibiotics [4–6].

Currently, the best line of defence in treating bacterial infections is the accurate diagnosis of the causative agent and knowledge of the corresponding antimicrobial susceptibility profiles of the pathogen. Clinical veterinary microbiology employs both culture-based and rapid automated methodologies such as MALDI-TOF for accurate bacterial identification [7]. Identification of a bacterial isolate is necessary, but without accurate and comprehensive antimicrobial susceptibility testing results, it is not sufficient for determining an effective treatment plan. As an example, staphylococcal...
isolates from pustules on dogs with superficial bacterial folliculitis have been reported to be the same strain as indicated by PFGE, but these related isolates exhibited different antibiotic susceptibility profiles [8]. Bacterial infections from skin, wounds and ears can be complex when more than one organism is present, further complicating treatment [7]. These findings indicated that additional sampling should be conducted from more than one pustule if the initial antibiotic regimen is not successful in eliminating the infection, which may be comprised of multiple isolates with altered AMR that have the same PFGE genotype [8]. These studies illustrate the complex nature of successfully accessing the full spectrum of AMR in a mixed population of bacteria in order to determine the most effective antibiotic treatment strategy.

Some infections in human medicine have been described as polymicrobial in composition and often contain genotypic and phenotypic variants that arise during growth as a biofilm-associated infection (reviewed in [9, 10]). Antibiotic treatments are ephemeral at best when targeting complex polymicrobial infections such as those found in cystic fibrosis patients [11]. A better understanding of the genotypic and phenotypic diversity that contributes to variation in antimicrobial susceptibility of bacterial strains residing within an infection will allow for improved diagnosis and more precise treatment of the bacterial infection.

One mechanism by which microbes tolerate and resist antibiotic treatment is through the production of a biofilm, which is a community of bacteria embedded in self-produced extracellular polymeric substances (EPS). Although the composition of biofilm EPS is known to include exopolysaccharides, nucleic acids, lipids and proteins, the exact composition varies among different species and strains of bacteria. An important role of the EPS matrix, which encapsulates the bacteria, is to provide a protective coating to aid in colonization and persistence [12]. Bacteria within a biofilm respond to environmental stress and adapt accordingly through physiological adaptation and mutation to alter phenotypes that favour survival (reviewed in [13]). Accordingly, growth in a biofilm generates phenotypic diversity and selects for colony morphology variants when grown on agar-based medium, which include bacterial colonies that appear rough, wrinkly or even as small colony variants as compared to the parental strain [14]. In the veterinary medical field, biofilms have been reported to exist in canine ears, canine dental plaque, equine limbs and the equine uterus [15–19]; however, detailed investigations about phenotypic and genetic diversification of the bacteria residing in biofilm communities and the role of signalling molecules in these biofilms has not been conducted.

Cyclic di-GMP, a nearly universal bacterial second messenger, is a signalling molecule that regulates biofilm formation in *P. aeruginosa* [20]. Cyclic di-GMP is synthesized from two molecules of GTP by diguanylate cyclase enzymes [21]. *P. aeruginosa* colony morphology variants, such as the CF39s and Δ*wspF* mutant of *P. aeruginosa* PAO1, constitutively express high levels of cyclic di-GMP and correspondingly exhibit increased tolerance to antibiotics, enhanced biofilm formation, increased Congo red binding and produce high levels of the CdrA adhesin [20, 22, 23]. Conversely, cyclic di-GMP is degraded to pGpG intermediates or directly to two GMP molecules by phosphodiesterases, hydrolases or oligoribonucleases [24–27].

In this study, *P. aeruginosa* veterinary clinical isolates were supplied by the Colorado State University Veterinary Diagnostic Laboratories (CSU VDL). We investigated how distinct isolates from the same clinical sample differed using various phenotypic and genotypic assays that characterized colony morphology, antibiotic susceptibility, motility, cyclic di-GMP levels and biofilm formation in addition to the genetic relatedness of the isolates. We hypothesized that some of these differences among the clonal isolates are a result of alteration of cyclic di-GMP-mediated signalling. Towards this aim, cyclic di-GMP signal accumulation was evaluated with a cyclic di-GMP-responsive reporter assay. Additionally the levels of cyclic di-GMP in the isolates were respectively increased and decreased by the conditional expression of the diguanylate cyclase encoded by PA1120 (*tpbB*) and the phosphodiesterase encoded by PA2133 to further evaluate the role of cyclic di-GMP. The genetically related bacterial isolates representing clonal lineages were characterized to determine the potential of biofilm-associated infections to generate phenotypic variants with altered biofilm-forming capabilities and antibiotic susceptibility profiles.

**METHODS**

**Collection of clinical isolates**

*Pseudomonas aeruginosa* isolates used in this study were obtained from samples submitted for diagnostic analysis to the CSU VDL and Equine Reproduction Laboratory between April 2012 and November 2013. Eighteen samples were processed according to standard operating procedures of the CSU VDL. Briefly, samples were incubated at 37°C on TSA with 5% sheep blood and MacConkey agar (Thermo Scientific). Colonies from TSA or MacConkey with visual characteristics consistent with *P. aeruginosa* were isolated and identified using routine procedures. The 38 *P. aeruginosa* isolates used in this study were isolated from samples that originated from numerous infection sites including equine nares, equine uterine lavage, feline nares, feline abscesses, canine ears, canine vulva, canine urine, canine skin abscesses, canine bronco-alveolar lavage and a canine hip joint. MALDI-TOF analysis, as previously described [15], was utilized as an additional means to confirm the identity of isolates numbered 42121A and 42121B as well as 83468A and 83468B as *P. aeruginosa*, because of the abnormal morphologies associated with these strains. Bacterial cultures were routinely grown in Lysogeny Broth (LB-Lennox) and incubated in a shaking incubator at 37°C for 24 h. After receiving the isolates, all were frozen at −80°C.
Identification of colony morphology variants

Once isolated, the bacteria were characterized based on the colony morphology observed on Vogel Bonner minimal medium (VBMM) supplemented with 40 µg ml⁻¹ Congo red, 15 µg ml⁻¹ brilliant blue and 10 g l⁻¹ Noble agar. VBMM was made by adding the following components, in order, to 800 ml of ddH₂O: 2.0 g MgSO₄·7H₂O, 20 g citric acid, 35 g NaNH₂HPO₄·4H₂O and 100 g K₂HPO₄. After adjusting pH to 7.0, water was added up to 1 l and filter sterilized [28]. Overnight growth of control isolates, PAO1 and PAO1 Δ wspF, and clinical veterinary isolates 42121A, 42121B, 83468A and 83468B were struck for isolation onto VBMM agar plates containing Congo red and brilliant blue. The identity of the colony morphology variants was verified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis as previously described [15]. The plates were incubated for 24 h at 37 °C and then moved to 28 °C for an additional 24 h. After incubation, the colony morphology of individual colonies was observed and images were captured on a Leica MZ95 microscope.

Congo red and brilliant blue dye binding

P. aeruginosa veterinary clinical isolates were grown on VBMM with 40 µg ml⁻¹ Congo red and 15 µg ml⁻¹ brilliant blue and 10 g l⁻¹ Noble agar. A 3 µl sample of an overnight culture was spotted and then incubated at 37 °C for two days.

Swim and twitch motility

Each isolate was tested for motility in swimming and twitching assays. For swimming (flagella-mediated) motility, 2 µl of the overnight cultures were stab inoculated into low-density LB agar (0.3 %) medium. Plates were incubated right side up for 24 h at 37 °C. After incubation, swim diameters were measured. Each isolate was done in triplicate. For twitch (pili-mediated) motility, a disposable inoculating needle was dipped into overnight cultures and then pierced to the bottom of an LB plate. The plates were incubated at 37 °C for 24 h, LB agar was removed and the adherent bacteria were stained with 2 ml 0.05 % crystal violet for approximately 5 min. Plates were washed twice with 2 ml of PBS, allowed to dry and diameters of twitch zone growth were measured. Each isolate was evaluated in triplicate.

Monitoring cyclic di-GMP levels

P. aeruginosa PA01, PAO1 Δ wspF and veterinary clinical isolates 42121A, 42121B, 83468A and 83468B were transformed by electroporation with pCdrA::GFP [29], which is a fluorescent-based reporter of intracellular levels of cyclic di-GMP. Transformants were selected on LB agar supplemented with 100 µg ml⁻¹ gentamicin. Overnight cultures were grown in LB broth containing 100 µg ml⁻¹ gentamicin, then 10 µl was added to 1 ml LB broth with no antibiotic, and incubated for 4 h. After incubation, 1 ml from the diluted culture was spun down at 7000 g for 2 min and the supernatant was removed. Pellets were gently washed in 1 ml PBS, centrifuged at 7000 g for 2 min and the supernatant was removed. The resulting pellets were resuspended in 1 ml PBS, then 100 µl of each resuspended pellet was transferred into a black, clear bottom 96-well plate (Greiner Bio-One #655096). The OD₆₀₀ and OD₄₉₀ values were measured on a Perkin Elmer Enspire, and a ratio of the fluorescence (OD₄₉₀) divided by the absorbance (OD₆₀₀) was calculated.

Static biofilm assay

Quantitative analysis of biofilm formation was conducted as previously described [30] with the following minor modifications. Overnight cultures of P. aeruginosa PA01, PAO1 Δ wspF and veterinary clinical isolates 42121A, 42121B, 83468A and 83468B were grown in LB and subsequently diluted to a final OD₆₀₀=0.1. Each well of a 96-well plate (Nunc Microwell 96-well microplates #243656, Thermo Scientific) was inoculated with 100 µl of bacterial cultures and replicated in five wells. The plates were statically incubated for 24 h at 37 °C. The bacterial culture was pipetted out of the wells and the wells were rinsed with 125 µl of PBS. After the initial rinse, 125 µl of 0.05 % crystal violet was added to each well and incubated at room temperature for 15 min. Wells were gently rinsed twice with 200 µl PBS, then 300 µl 95 % ethanol was added to each well and incubated at room temperature for 30 min. The solubilized dye was thoroughly resuspended by mixing and 200 µl was transferred to a new 96-well plate. Absorbance (OD₆₀₀) was measured on a Perkin Elmer Enspire plate reader.

Alteration of cyclic di-GMP levels

Cyclic di-GMP levels were respectively increased or decreased through conditional expression of the diguanylate cyclase PA1120 (tpbB) and the phosphodiesterase PA2133 as previously described [20, 31]. P. aeruginosa PA01, PAO1 Δ wspF, 42121A and 42121B were transformed with either pN105 (empty vector), pN2133 (arabinose-inducible phosphodiesterase, PA2133) or pN1120 (arabinose-inducible diguanylate cyclase, PA1120). Transformed strains were maintained on 100 µg ml⁻¹ gentamicin. Enzymatic activity of either PA2133 (phosphodiesterase activity) or PA1120 (diguanylate cyclase) was conditionally induced using 0.5 % l-arabinose. Analysis of colony morphology and biofilm formation was performed as described above.

gyrA sequencing

Genomic DNA from PA01, PAO1 Δ wspF, 42121A and 42121B was extracted by Qiagen DNeasy Blood and Tissue Kit. Sequencing primers for gyrA were forward (5’-TTC TCCCCGTCATAATCGAA-3’) and reverse (5’-TGTTCA ACTGGTAGGCA-3’). gyrA sequencing results from 42121A and 42121B were aligned to gyrA from PA01 (taxid: 208964).

RAPD analysis

Genomic DNA was extracted from 42121A, 42121B, 83468A, 83468B, PAO1 and PAO1 Δ wspF using the Qiagen DNeasy Blood and Tissue Kit. RAPD PCR was conducted using primer 272 (3’-AGCGGGCACA-5’) [32]. The PCR mixture
included GoTaq DNA polymerase colourless mastermix (Promega), 40 ng genomic DNA and 25 mM MgCl₂. The cycling conditions for PCR reactions were 95 °C for 2 min, 3 cycles of 94 °C for 1 min, 56 °C for 2 min, 72 °C for 2 min, followed by 29 cycles at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min with a final extension at 72 °C for 5 min. The PCR products were resolved on a 1.5 % agarose gel.

**Kirby–Bauer disk diffusion susceptibility test**

Strains were struck onto LB plates and incubated overnight at 37 °C. Bacterial colonies were suspended in 1 ml of a saline solution using the BD BBL Prompt Inoculation System. A cotton swab was dipped in the saline solution containing the bacteria. The bacterial solution was spread for confluence on Mueller–Hinton (MH) agar plates. A Kirby–Bauer disc dispenser was used to dispense eight antibiotic disks containing various antibiotics onto the inoculated MH agar plates. The eight antibiotic disks (BD BBL Sensi-discs) contained: ciprofloxacin (disk potency of 5 µg), ticarcillin/ clavulanic acid (75 µg/10 µg, respectively), penicillin (10 U), gentamicin (10 µg), amikacin (30 µg), enrofloxacin (5 µg), and ceftiofur (30 µg). Plates were incubated at 37 °C for 24 h and zones of inhibition were measured. Results were interpreted as susceptible, intermediate or resistant to the drug based on published CLSI zone diameter standards [33].

**Determination of MICs**

Overnight cultures were grown in LB and 100 µl was transferred into 2.9 ml MH broth (MHB) and incubated in a 37 °C shaking incubator until the OD₆₀₀ reached 0.6–0.8 (~2 h). Cultures were diluted to a final OD₆₀₀ of 0.1 in a sterile saline solution and 200 µl was transferred to 9.8 ml MHB. The culture solution was mixed with 11 mg ml⁻¹ resazurin (Sigma) solution at a dilution of 20 µl of resazurin (also known as alamar blue) solution per ml [34, 35]. A 50 µl aliquot of mixed solution was added to each well of a 96-well plate containing 50 µl of each antibiotic diluted in MHB at the defined concentrations. For control wells, MHB (no bacteria) was mixed with resazurin. The plate was then statically incubated for 24 h at 37 °C. The colour of the resazurin indicator was used to identify antibiotic concentrations that inhibited bacterial growth. Resistant and metabolically active bacterial cells were observed as pink wells, whereas susceptible and metabolically inactive bacterial cells were indicated by blue wells.

**Statistics**

Statistics were performed using GraphPad Prism 6 software. Student's t-tests were run between paired isolates for the biofilm, motility and cyclic di-GMP reporter assays to determine whether the values were significantly different. A critical P-value of 0.01 was used.

**RESULTS**

**Identification of paired colony morphology variants**

Bacterial isolates from samples that were submitted from referring veterinarians to CSU VDL for culture were isolated on TSA with 5 % sheep red blood and MacConkey agar (Fig. 1a). During the course of these studies, we were blinded to the history of the patients and unable to obtain information concerning antibiotic use during treatment of the infection, or the ultimate fate of the animals from which the bacterial isolates were obtained. Additionally, we did not determine whether the isolated bacterial strains were virulent or causal to infection. Individual colonies of the bacteria...
isolated on TSA or MacConkey agar plates with visual characteristics consistent with \textit{P. aeruginosa} were grown on Vogel–Bonner minimal media supplemented with citrate, Congo red, brilliant blue and Noble agar (VBMM CRBB) to selectively identify \textit{P. aeruginosa} colony morphology variants. A mixed population of \textit{P. aeruginosa} colony morphology variants can be observed using this technique as demonstrated by 42121A and 42121B, which were struck onto a VBMM CRBB agar plate to demonstrate the ability of this culture-based approach to differentiate colony morphology phenotypes (Fig. 1b).

A total of 17 paired and one quadruplet set of \textit{P. aeruginosa} isolates originally isolated and cultured from individual samples submitted to CSU VDL were spotted on VBMM plates containing Congo red and brilliant blue to assess exopolysaccharide production, an indicator of biofilm-formation capacity (Fig. 1c). PAO1 and PAO1 $\Delta$wspF served as non-binding and hyper-binding controls for dye binding, respectively (Fig. 1c). We identified two pairs of \textit{P. aeruginosa} isolates (42121A/42121B and 83468A/83468B) from samples obtained from canine ears that exhibited contrasting colony morphologies. Each pair of isolates was cultured from a single sample and one member of each pair exhibited enhanced Congo red and brilliant blue dye binding, whereas the corresponding partnered isolate did not [Fig. 1 and Table S1 (available in the online Supplementary Material)].

We observed stark differences between the two control strains as well as between the paired isolates on VBMM CRBB agar plates. The laboratory control strain, PAO1 (Fig. 2a), exhibited a larger colony size and less dye binding than its hyper-binding rugose (wrinkled) counterpart, PAO1 $\Delta$wspF (Fig. 2b), a strain that is known to produce

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\textbf{Fig. 2.} Colony morphologies of control strains and two clonal pairs of veterinary clinical isolates. (a) PAO1, (b) PAO1 $\Delta$wspF, (c) 42121A, (d) 42121B, (e) 83468A and (f) 83468B. The small colony variant phenotype shows increased binding to the Congo red and brilliant blue dye, indicating increased amounts of exopolysaccharide production. Scale bar=2 mm.
Table 1. Antibiotic susceptibility of 42121A, 42121B, 83468A, 83468B, PAO1 and PAO1 ΔwspF using Kirby–Bauer disk diffusion assays

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>42121A</th>
<th>42121B</th>
<th>83468A</th>
<th>83468B</th>
<th>PAO1</th>
<th>PAO1 ΔwspF</th>
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<tr>
<td>Ciprofloxacin</td>
<td>R</td>
<td>R</td>
<td>S</td>
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<td>Ticarcillin</td>
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<td>Sulfamethoxazole</td>
<td>R</td>
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<td>Penicillin</td>
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<td>Gentamicin</td>
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<td>Amikacin</td>
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<td>Enrofloxacin</td>
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<td>Ceftiofur</td>
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R=resistant, I=intermediate and S=susceptible.

To determine whether the 42121 and 83468 paired isolates were genetically related, RAPD PCR using a single primer to amplify random segments throughout the genome was conducted to compare genomic DNA from 42121A and 42121B as well as 83468A and 83468B along with controls, PAO1 and PAO1 ΔwspF. RAPD banding patterns indicated that each group of pairs, 42121A/42121B, 83468A/83468B and PAO1/PAO1 ΔwspF, are genetically related to each other and each pair comprised a clonal lineage (Fig. S1).

Antibiotic susceptibility of clonal isolates

Antibiotic susceptibility profiles of the clonal pairs, 42121A/42121B, 83468A/83468B and PAO1/PAO1 ΔwspF, were evaluated to determine antimicrobial susceptibility profiles using the Kirby–Bauer agar disk diffusion method. Isolates 42121A and 42121B were resistant to the two fluoroquinolones (ciprofloxacin and enrofloxacin). Isolate 83468A exhibited intermediate resistance to enrofloxacin and 83468B was susceptible to enrofloxacin. Isolates 83468A and 83468B were susceptible to ciprofloxacin (Table 1). All strains were susceptible to the aminoglycoside, gentamicin, with the exception of 42121B which was intermediate in resistance (Table 1). All strains tested were resistant to sulfamethoxazole and penicillin (β-lactam) but susceptible to ticarcillin (β-lactam) and amikacin (aminoglycoside) (Table 1). Both of the clonal pairs, 42121A/42121B and 83468A/83468B, were resistant to ceftiofur (cephalosporin), whereas PAO1 and PAO1 ΔwspF exhibited susceptible and intermediate resistance phenotypes to ceftiofur, respectively (Table 2). Overall, the antimicrobial susceptibility observed in the disk diffusion assays was identical among the clonal isolates that comprised each pair, with the exception of gentamicin and enrofloxacin (Table 1).

To obtain additional quantitative data on the antimicrobial susceptibility profiles, the MICs (Table 2) of ciprofloxacin and gentamicin were determined for 42121A, 42121B and the control strains (PAO1 and PAO1 ΔwspF). Strains also were tested against amikacin as a susceptible control to evaluate the MIC assays. All four strains were susceptible to amikacin in the Kirby–Bauer assay and this result was confirmed with the resazurin MIC assay (Tables 1 and 2). Consistent with the Kirby–Bauer assay, 42121A and B were resistant to ciprofloxacin although the MIC value was higher for 42121B (80 µg ml⁻¹) than for 42121A (50 µg ml⁻¹), while PAO1 and PAO1 ΔwspF exhibited a much lower MIC value of 2 µg ml⁻¹ (Table 2). The MIC values observed for gentamicin were 5, 10 and 25 µg ml⁻¹ for PAO1, PAO1 ΔwspF and 42121A, respectively (Table 2). The gentamicin MIC value obtained for 42121B was nearly twice the concentration of the 42121A MIC, confirming the intermediate resistance phenotype observed for 42121B during evaluation with the Kirby–Bauer disk diffusion method.

Biofilm formation differs significantly among clonal isolates

Hyper-binding of Congo red and brilliant blue by P. aeruginosa indicates an increased production of the Pel and Psl exopolysaccharides and is an indicator of biofilm-forming potential [36]. Isolate 42121A exhibited a 9.1-fold increase in biofilm production as compared to 42121B, while isolate 83468B had a 3.8-fold increase in biofilm production compared to 83468A in a static microtitre dish biofilm assay (Fig. 3a). The hyper-biofilm former, PAO1 ΔwspF, had a 3.3-fold increase in biofilm production as compared to...
Fig. 3. Biofilm formation, swimming and twitching motility of clonal isolates. (a) Static biofilm formation was quantified after 24 h at 37°C. Paired isolates representing clonal lineages were also evaluated in swim (b) and twitch (c) motility assays. A critical $P$-value of $P<0.01$ was used to determine statistically significant differences in biofilm formation, swim zone and twitch zone diameters.
elevated levels of cyclic di-GMP as compared to 42121B. The levels of GFP expression measured for 42121A were significantly higher than the values measured for 42121B, suggesting that some of the phenotypes observed with 42121A might be due to elevated cyclic di-GMP levels similar to what was observed with PAO1 ΔwspF versus PAO1 (Fig. 4). No differences in pCdrA::GFP reporter activity were noted with 83468A and 83468B (Fig. 4).

**Depletion of cyclic di-GMP decreases biofilm formation and reverses colony morphology phenotype of 42121A**

To determine whether the biofilm and colony morphology phenotypes of 42121A and 42121B were dependent on cyclic di-GMP levels, we used arabinose-inducible expression of either PA1120 or PA2133, which encode cyclic di-GMP metabolic proteins. It should be noted that the colony morphologies of 42121A and 42121B were stable under the conditions tested, unless PA1120 or PA2133 was expressed from plasmids that were introduced to reverse colony morphology. Overexpression of PA2133, which encodes a phosphodiesterase that degrades cyclic di-GMP [20, 38], in either PAO1, PAO1 ΔwspF or isolate 42121A resulted in a significant decrease in biofilm production, suggesting that cyclic di-GMP levels contribute to the biofilm phenotypes in these isolates (Fig. 5a). The hyper-dye binding and small colony variant phenotype of 42121A was reversed with overexpression of the phosphodiesterase, PA2133 (Fig. 5b).

**Minimal differences in motility were observed between paired isolates**

In order to characterize additional phenotypic differences, two modes of motility were evaluated for the 42121 and 83468 isolate pairs. Flagella-mediated motility was measured in swimming motility assays, while pili-mediated motility was measured in twitching motility assays. There were no statistical differences detected between 83468A and 83468B or between 42121A and 42121B in swim zone diameters produced (Fig. 3b). However, 83468A (low biofilm producer) and 83468B (high biofilm producer) were statistically different in the twitch motility assay (Fig. 3c). There was no difference detected between 42121A and 42121B in the twitch assay. As anticipated, the laboratory strains, PAO1 and PAO1 ΔwspF, produced statistically significant differences in both swim and twitch diameters [37] (Fig. 3b, c).

**Cyclic di-GMP levels are elevated in 42121A**

Cyclic di-GMP levels were compared among isolates using a previously published cyclic di-GMP-responsive reporter plasmid, pCdrA::GFP [29]. The colony morphology variants and control strains (42121A, 42121B, 83468A, 83468B, PAO1 and PAO1 ΔwspF) were transformed with the pCdrA::GFP reporter (Table S1). Based on the phenotypic data from the 42121 pair of clonal isolates, we hypothesized that 42121A, a hyper-biofilm producer, would produce
et al. [39] to be linked to ciprofloxacin resistance in *P. aeruginosa* grown in the presence of sub-inhibitory concentrations of ciprofloxacin [39]. Three single nucleotide polymorphisms that did not alter the amino acid, C396T (histidine), C531T (glycine) and T780C (isoleucine), were also identified (Table 3). Interestingly, no mutations in the most common sites, Thr83 and Asp87 of GyrA [40], were detected.
DISCUSSION

In this study, we have shown that during diagnostic sampling of bacteria from clinical veterinary samples it is possible to identify colony morphology variants of *P. aeruginosa*. The observed differences in the altered colony morphology and biofilm-forming capacity of two related strains, 42121A and 42121B, were associated respectively with increased and decreased levels of cyclic di-GMP levels in two of the colony morphology variants described in this study. These variants differed in the amounts of exopolysaccharides that they produced as determined by Congo red and brilliant blue dye binding assays, which also correlated with the amount of biofilm produced by each strain. 42121A produced more biofilm and had significantly higher levels of the second messenger cyclic di-GMP as compared to 42121B. Interestingly, the hyper-biofilm formation phenotype observed for 42121A could be reduced with expression of a phosphodiesterase, suggesting that the levels of cyclic di-GMP produced by this strain were at least partially responsible for the biofilm-associated phenotypes observed in this study. Previous studies of colony morphology variants of *P. aeruginosa* have shown that point mutations and deletions can generate stable variants that have altered levels of cyclic di-GMP during growth as biofilm [20, 23]. Elevated levels of cyclic di-GMP have also been measured in colony morphology variants isolated from human clinical cases [23, 41].

Both of the 42121A and 42121B isolates exhibited decreased swimming motility as compared to PAO1 or the hyper-biofilm producer, PAO1 Δ*wspF* (Fig. 5). The loss of swimming motility by both isolates from the same sample might be indicative of an adaptation to evade the surveillance of the host immune system. Previous studies using *P. aeruginosa*, *Eschericia coli* and *Vibrio cholerae* have demonstrated that loss of flagellum, which is a ligand for the innate immune system, confers decreased phagocytosis [42, 43]. Future studies will evaluate which flagellar components have been altered in 42121A or 42121B.

One hypothesis that accounts for the observation of phenotypic diversity during the isolation of colony morphology variants from clinical samples is the ‘insurance effects hypothesis’ [44]. Boles *et al.* previously demonstrated that bacteria grown as a biofilm generate multiple types of colony morphology variants that have adapted to possess either accelerated detachment or hyper-biofilm production, which cooperatively function together during biofilm growth to benefit bacterial persistence [44]. In the simplest scenario of the insurance effects hypothesis, 42121A is the cornerstone of the biofilm, being a hyper-biofilm producer, while 42121B has a complementary role that allows for rapid detachment and dispersal of the biofilm which would facilitate dissemination during the infection. Additional environmental stressors, such as the addition of antibiotics, can further drive genetic diversification and corresponding alterations in phenotypes, especially when antibiotics are administered at sub-inhibitory concentrations [39].

The identification of hyper-biofilm-forming variants during clinical diagnostics is important because of the established link that exists between increased antibiotic tolerance, biofilm formation and the corresponding treatment failure from biofilm-associated chronic infections [15, 45, 46]. *P. aeruginosa* hyper-biofilm-forming variants with elevated tolerance to the antibiotic tobramycin have previously been identified [47]. If hyper-biofilm-forming colony morphology variants are not accounted for during isolation and antibiotic susceptibility screening in the diagnostic laboratory, then the clinician will develop a treatment strategy that may be ineffective.

The characterization of pairs of clonal isolates that have adapted multiple mechanisms for antibiotic tolerance and resistance provides additional insight into considering the appropriate treatment strategy. As identified in this study, one pair of clonal isolates (42121A and B) included a hyper-biofilm-forming strain and a corresponding hypobiofilm-forming strain. In a chronic infection, the hyper-biofilm-forming strain (42121A) would be expected to be more tolerant to a broad range of antimicrobial treatments *in vivo*. Interestingly, 42121B demonstrated elevated levels of resistance to amikacin, gentamicin and ciprofloxacin during MIC testing of planktonic bacteria cells as compared to 42121A. It is possible that this difference may be indicative of the evolution of additional mechanisms of antibiotic resistance during prolonged antimicrobial treatment in a strain that is deficient for biofilm production. Although the phenotypic differences between 83468A and 83468B were much more subtle as compared to 42121A and 42121B, we hypothesize that the differences observed in twitch motility between 83468A and 83468B may contribute to the differences observed for the colony morphology phenotype in this clonal pair of isolates. Future efforts will focus on identifying the genetic differences that contribute to the observed phenotypic differences in biofilm formation and antibiotic resistance in these clonal isolates, to better understand how bacteria undergo diversification during biofilm-associated infection and antibiotic treatment. It should be noted that we did not observe reversion under the conditions tested in our assays using rich growth media (e.g. LB). However, future experiments will specifically challenge colony morphology variants to observe any potential phenotypic reversion that may arise under varying conditions, which could include growth in a biofilm or minimal medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>gyrA (nucleotide)</th>
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<tbody>
<tr>
<td></td>
<td>248</td>
</tr>
<tr>
<td>PAO1</td>
<td>C</td>
</tr>
<tr>
<td>42121A</td>
<td>T</td>
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<tr>
<td>42121B</td>
<td>T</td>
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Antibiotic resistance in bacterial isolates from human infections has been well documented [1, 48]; however, significantly less is known about antibiotic resistance of bacterial isolates from companion animals although there is evidence that it is on the rise in veterinary medicine as well [49, 50]. The percentage of cultures from the canine ear that tested positive for *P. aeruginosa* varied between 21 and 31 from 1992 to 1997 [51]. A recent study classified nearly 40% of *P. aeruginosa* isolates from dogs with otitis media as biofilm-formers, demonstrating the potential contribution of biofilm formation in these infections [18]. Increased antibiotic resistance of bacterial isolates, paired with a decreased availability of effective antibiotics, significantly limits the treatment regimens that veterinarians and human doctors can prescribe. Collecting detailed information about the biofilm-forming potential and antimicrobial susceptibilities of the bacteria associated with the infection will allow doctors to prescribe a more precise course of treatment to eradicate the infection and minimize relapse. Furthermore, diagnostics should evaluate antibiotic susceptibility profiles in a broader context that more accurately evaluates the genotypic diversity of the bacterial population that resides within an infection site as opposed to a single isolate from that population.

In this study, we identified strains exhibiting high levels of resistance to fluoroquinolone antibiotics. Fluoroquinolones, such as ciprofloxacin and enrofloxacin, are clinically relevant veterinary drugs used to treat *P. aeruginosa* infections in companion animals. Enrofloxacin was the first fluoroquinolone approved for veterinary use and de-ethylation produces its active metabolite, ciprofloxacin [52, 53]. In 2008, ciprofloxacin resistance in *P. aeruginosa* isolates was reported to be 16% [54], but a more recent study in 2015 with isolates collected from 2008 to 2011 in France concluded that 63% of *P. aeruginosa* isolates from dogs were resistant to ciprofloxacin [55]. Increased administration of fluoroquinolones in the veterinary field parallels the increase in the number of ciprofloxacin-resistant infections reported in human medicine [56]. Understanding the mechanisms of fluoroquinolone resistance in bacteria from companion animals is important because the same fluoroquinolones are prescribed in human medicine [54] for *P. aeruginosa* infections that can be transmitted between companion animals and their owners [57–59]. Antibiotic resistance in zoonotic pathogens from companion animals should be closely monitored, especially in cases where the owner may be immunocompromised [60, 61].

There has been significant emphasis placed on emerging resistance to frontline antibiotic therapies in veterinary practice; however, the underlying mechanism(s) for the emergence of resistance has not been fully addressed. *P. aeruginosa* veterinary isolates resistant to numerous antibiotics such as β-lactams, sulfonamides and fluoroquinolones have previously been reported [55, 62]. In this report, we have added antibiotic tolerance mediated by cyclic di-GMP to the list of potential mechanisms. We demonstrated that cyclic di-GMP levels that alter biofilm formation and colony morphology is also a plausible mechanism that should be addressed in recurring or recalcitrant veterinary infections that may contribute to the further development and spread of antibiotic resistance.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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