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Staphylococcus pseudintermedius Isolated from Two Dog Cases with Ophthalmic Lesions

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Abstract Two dog cases with history of recurrent bacterial conjunctivitis were submitted to Purdue University for bacterial culture and sensitivity testing. The bacterial cultures were also tested for their ability to form a biofilm. Biofilm forming *Staphylococcus pseudintermedius* was isolated from the two cases and identified by Vitek2 and PCR analysis. The bacterial antibiotic sensitivity testing revealed that Isolate one was susceptible to ticarcillin, ceftiofur, cefazolin, moxifloxacin, amikacin and rifampicin with MICs ranging 0.03125-4 µg/ml. Isolate two was susceptible to ceftiofur, gatifloxacin, cefazolin, moxifloxacin, tetracycline, doxycycline and rifampicin with MICs ranging <0.0625–4 µg/ml. Meanwhile, the biofilm antibiotic sensitivity testing revealed that Isolate one biofilm was resistant to all antibiotics except for ceftiofur and moxifloxacin with MICs <0.25 and 8 µg/ml respectively. Isolate two biofilm was resistant to all antibiotics except for tetracycline, amikacin, doxycycline, chloramphenicol and rifampicin with MICs ranged from <0.125 to 8 µg/ml. Case one was resolved with topical cefazolin therapy. Meanwhile case two showed complete recovery after treated with topical levofloxacin plus oral doxycycline.

Keywords: *Staph. pseudintermedius, biofilm, dogs, ophthalmic lesions*

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1. Introduction

Staphylococcus pseudintermedius, the prevalent species in the *Staphylococcus intermedius* group, is an opportunistic pathogen recognized as the leading cause of skin, ear, and post-operative bacterial infections in dogs and cats [1,2]. While knowledge of the pathogenesis of *S. pseudintermedius* is limited, it is known that veterinary strains are able to produce numerous virulence factors, including β-hemolysin, clumping factor, coagulase, DNase, protein A, lipase, leukotoxin, exfoliative toxin, and enterotoxins [3,4]. Furthermore, for veterinary dermatologists in particular, *S. pseudintermedius* methicillin-resistant strains have recently emerged as a major challenge because of extensive multidrug resistance and their behavior as nosocomial pathogens [5]. Biofilm formation is considered to be one of the most important virulence factors in staphylococci. The biofilm-forming ability of veterinary *S. pseudintermedius* isolates has been reported in a limited number of cases [3,6,7]. Strains of *S. pseudintermedius* from canine bacterial conjunctivitis have also demonstrated increased biofilm production [3].

Case 1 Description: A 10-year-old female neutered Cairn terrier was initially presented to the North Central Veterinary Emergency Clinic, Westville, IN for ophthalmic examination for chronic severe keratoconjunctivitis sicca (KCS). Therapy with tacrolimus 0.02% was initiated. Two years after the initial examination, bacterial conjunctivitis occurred in the right eye (OD). A conjunctival swab was collected from the OD. A *Staphylococcus pseudintermedius*, sensitive to amikacin, ticarcillin, and cefazolin was detected. Therapy was started with topical amikacin q6h and dilute betadine solution q12h. The conjunctivitis resolved. Over the next two year the conjunctivitis recurred periodically OD and resolved when treated with tobramycin q6h and flushing with dilute betadine solution q12h. Twenty-two months after the first culture, a conjunctival swab was collected from the right eye and submitted to Purdue University for bacterial culture and sensitivity testing.

Case 2 Description: A 5-year-old female neutered Yorkshire terrier was initially presented to Animal Eye Care, Bellingham WA for ophthalmic examination, two weeks following traumatic proptosis of the right eye. At time of proptosis, the referring veterinarian surgically repositioned the globe and placed temporary tarsorrhaphy sutures in the eyelids. Upon removing the sutures two weeks later, corneal ulceration was noted, prompting patient referral to Animal Eye Care. Ophthalmic examination of the left eye was unremarkable. Evaluation of the right eye revealed corneal ulceration, suspect bacterial conjunctivitis, absence of corneal sensation, and blindness secondary to optic nerve trauma. The right eye was subsequently enucleated by the referring veterinarian.

However, two months later the dog was presented to Animal Eye Care for ophthalmic examination of the remaining left eye, with a seven-week history of purulent ocular discharge and blepharospasm. KCS with suspect secondary bacterial conjunctivitis were diagnosed. Topical therapy with ofloxacin and ketorolac solutions, tacrolimus 0.03% ointment, and sodium hyaluronate 1% gel was initiated. Reexamination two months later revealed that ocular discharge had initially improved, but was recurring. Blepharospasm had improved but was not resolved. The KCS was uncontrolled. Because prescribing topical tacrolimus 1% solution and neomycin/polymyxin/bacitracin ointment did not resolve ocular discharge and discomfort after two months of therapy, the ocular discharge was cultured at a local diagnostic laboratory, yielding methicillin-resistant *S. pseudintermedius (MRSP).* Following consultation by the secondary author with the fourth author, the bacterial colony cultured from the initial conjunctival swab was submitted to Purdue University for evaluation.

The bacterial cultures from both cases were also tested for their ability to form a biofilm. Antibiotic sensitivity assays were performed with both the planktonic bacteria and with the biofilms.

2. Materials and Methods

2.1. Samples and Media

Bacterial colonies harvested from conjunctival swabs from both dogs were included in the study. Clinical specimens were inoculated onto 5% sheep blood agar and incubated at 37°C for 18–24 hours, and the grown isolates identified at the Indiana Animal Disease Diagnostic laboratory to the species level using the Vitek 2 (BioMérieux). Vitek identification of *S. pseudintermedius* was confirmed by a positive coagulase test as described before [8]. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from Sigma-Aldrich while Trypticase soy broth (TSB) and Trypticase soy agar (TSA) were purchased from Becton, Dickinson (Cockeysville, MD).

2.2. Reagents and Antibiotics

Bacitracin (J62432; Alfa Aesar), rifampicin (R3501; Sigma), trimethoprim (T2286; TCI), gatifloxacin (15156; CHEM-IMPEX INT'L INC), chloramphenicol (C0378; Sigma), ofloxacin (O8757; Sigma), tetracycline (87128; Sigma), erythromycin (E5389; Sigma), cefazolin sodium salt (C2242; TCI), sulfamethoxazole (00821; CHEM-IMPEX INT'L INC), doxycycline monohydrate (J63805; Alfa Aesar), ceftiofur hydrochloride (AK-51338; Ark Pharm, Inc.), ticarcillin disodium salt (T36500-1.0; rpi Research Products International Corp.), moxifloxacin hydrochloride (AX8032396; OXCHEM), gentamicin sulfate (ALX-380-003; Enzo Life Sciences), neomycin sulfate (ALX-380-035; Enzo Life Sciences), amikacin (ALX-380-045; Enzo Life Sciences), polymyxin B sulfate (ALX-380-040; Enzo Life Sciences), tobramycin (ALX-380-018; Enzo Life Sciences) and lysostaphin (3,000 U/mg) from *Staphylococcus simulans* (L9043; Sigma).

2.3. Bacterial PCR Identification

2.3.1. Sampling and DNA Extraction

A loop of the isolated bacteria was taken with 200 µl of sterile PBS and 180 µl of DNA extraction buffer (DNeasy Blood & Tissue Kit by Qiagen) and subsequently following the protocol of the manufacturer. DNA was eluted with 100 µl of elution buffer and tested for PCR-amplifiable DNA.

2.3.2. 16S rRNA Gene Region Amplification

PCR reactions were conducted using universal bacterial primers to amplify the 16S rRNA genetic region. 5F forward primer 5 ′ - TTGGAGAGTTTGATCCTGGCTC - 3′ and 1194R reverse primer 5′- ACGTCATCCCCACCTTCCTC - 3′ were utilized to amplify the 16S rRNA region of bacterial DNA [9]. The 26-µl PCR reaction mixture contained 3 µl of DNA template, 0.5 µl of 100 mM forward and reverse primers, 13 µl GoTaq® Green Master Mix (Promega) and 9 µl nuclease-free water. The PCR was performed in a Bio-Rad thermal cycler using the following cycle condition: initial hold at 94°C for 5 min and then 30 cycles of denaturing at 94°C for 30s, annealing for 30s at 60°C, and extension at 72°C for 2 min. The reaction ended with a final extension at 72°C for 2 min and hold at 4°C.

2.3.3. Agarose gel Electrophoresis

Detection of PCR-amplified product was performed by electrophoresis on a 1% (wt/vol) agarose gel stained with ethidium bromide. A 1kb plus ladder (Invitrogen) was also run in parallel for approximate PCR product band sizing.

2.3.4. DNA Sequencing of PCR Products

Amplified PCR products were extracted from the gel using a Gel Extraction Kit from Qiagen. The purified unknown DNA fragments were sent for nucleotide sequencing at the Purdue Genomics Facility (ABI 3137XL low-throughput capillary machine) using the following primer 810R GGCGTGGACTTCCAGGGTATCT.

2.3.5. Fragment Analysis of the 16S rRNA Region

16S rRNA region rDNA sequences were analyzed by using the BLAST alignment program of the GenBank database (National Institutes of Health) (Bacterial BLAST).

2.4. Antibacterial Assays

Minimum inhibitory concentrations (MICs) of antibiotics were evaluated using the broth microdilution technique in MHB with an initial inoculum of 5×10^5 cells in non-treated Polystyrene microtiter plates (CELLTREAT Scientific Products, 229596) in accordance with the Clinical and Laboratory Standards Institute [10]. The MIC was interpreted as the lowest concentration of antibiotic that completely inhibited the visible growth of bacteria after 16 h of incubation of the plates at 37°C. Each agent was tested in triplicate in at least two independent experiments. The highest MIC value was reported.

2.5. Formation of Biofilms

The ability of the two *S. pseudintermedius* isolates to form biofilm was investigated by a method described by Stepanovic et al. [11] with some modifications. Bacteria were cultivated overnight in Tryptic Soy Broth (TBS) supplemented with 1% glucose. Each culture was diluted 1:100 in the same medium and, subsequently, 200 µL of the diluted bacterial suspension was transferred, in triplicates, into the wells of sterile 96-well polystyrene microtiter plates and incubated at 37°C for 24 h. The negative control contained only the growth medium. The plates were washed twice with 200 µL of PBS (pH 7.2) and dried at room temperature prior to adding 1% crystal violet solution for staining. The plates were incubated at room temperature for 15 min before excess dye was removed by washing with water. The biofilm-bound dye was dissolved in 150 µL of 95% ethanol. The OD of dye (represent biofilm density) were measured at 595-nm absorbance by using a microplate reader (Spectra Max i3x, 363701003).

2.6. Antibiotic Activity against Preformed Biofilms

The activity of antibiotics against 48 h-old biofilms was assessed by viable colony count. Biofilms were allowed to form in each well of a 96-well flat-bottom polystyrene tissue-treated microtiter plate (Becton, Dickinson and Company), as described above. Following 48 h-incubation, biofilms samples were washed twice with PBS, then exposed to 200 µl of drug-containing cation-adjusted Mueller–Hinton broth (CAMHB) (prepared with 1, 2, 4, 8, 16, 32, 64, and 128 × MIC of antibiotics). After incubation at 37 °C for 24 h, non-adherent bacteria were removed by washing twice with 200 µl sterile PBS, and biofilm samples were scraped as described above. Cell suspension was then vortexed for 1 min to break up bacterial clumps. Bacterial counts were performed by plating serial 10-fold dilutions of this suspension on MHA plates. Control biofilm samples were not exposed to antibiotics. Minimum Biofilm Eradication Concentration (MBEC) was calculated as the minimum concentration of tested antibiotic able to eradicate preformed biofilm.

3. Results

3.1. Culture Results

The bacterial colonies isolated from conjunctival swabs from the two dogs were tested and exhibited bacterial growths identified as *S. pseudintermedius* by Vitek 2 (BioMérieux). Vitek identification of *S. pseudintermedius* was confirmed by a positive coagulase.

3.2. PCR Results

Identification of the isolated bacterial pathogen via amplification of 16S rRNA gene region of bacterial DNA by conventional PCR resulted in successful identification of *S. pseudintermedius*. A PCR band at 310 bp was sequenced and the sequence was used to identify the pathogen using the bacterial BLAST program (Figure 1).

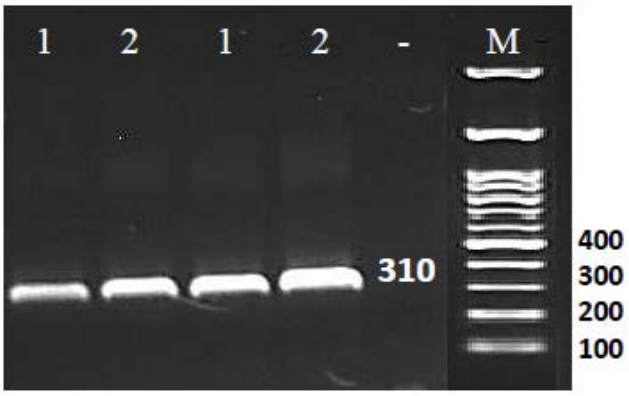

Figure 1. PCR products of the two *Staphylococcus pseudintermedius* isolates as determined by 1% agrose gel electrophoresis. M: 100 bp ladder, Lane 1, 2 the two isolates, and lane - is the negative control

3.3. Antibacterial Assays Results

Minimal inhibitory concentrations of antibiotics are listed in Table 1. Isolate one was susceptible to ticarcillin, ceftiofur, cefazolin, moxifloxacin, amikacin and rifampicin with MICs ranging 0.03125–4 µg/ml. Meanwhile it showed intermediate susceptibility to tobramycin, gatifloxacin, ofloxacin, trimethoprim, polymyxin B, neomycin, tetracycline, and chloramphenicol with MICs ranging 8-32 µg/ml. This isolate was resistant to gentamicin, bacitracin, sulfamethoxazole and erythromycin with MICs ranging 64– >128 µg/ml. Isolate two was susceptible to ceftiofur, gatifloxacin, cefazolin, moxifloxacin, tetracycline, doxycycline and rifampicin with MICs ranging <0.0625–4 µg/ml. Meanwhile it showed intermediate susceptibility to ticarcillin, tobramycin, ofloxacin, polymyxin B, amikacin and chloramphenicol with MICs ranging 8–32 µg/ml. This isolate was resistant to gentamicin, bacitracin, trimethoprim, neomycin, sulfamethoxazole and erythromycin with MICs ranging 64– >128 µg/ml.

Table 1. MIC (µg/ml) of the two isolates of *S. pseudintermedius* against antibiotics

	Isolate 1			Isolate 2		
	MIC	Biofilm MIC	MBEC	MIC	Biofilm MIC	MBEC
Ticarcillin	2	>128	>128	16	>128	-
Tobramycin	16	128	>128	32	>128	-
Ceftiofur	0.03125	<0.25	2	1	>128	-
Gatifloxacin	8	64	>128	4	64	>128
Gentamicin	64	>128	>128	128	>128	-
Ofloxacin	32	128	>128	16	32	
Cefazolin	0.125	32	128	0.5	128	-
Bacitracin	64	128	128	128	128	-
Trimethoprim	32	>128	>128	128	>128	-
Polymyxin B	8	32	>128	32	64	-
Neomycin	32	>128	>128	>128	>128	-
Tetracycline	32	64	128	<0.125	<0.125	32
Moxifloxacin	4	8	>128	2	64	>128
Amikacin	2	>128	>128	8	8	>128
Sulfamethoxazole	>128	>128	>128	>128	>128	-
Doxycycline	64	128	>128	0.25	0.25	>128
Erythromycin	>128	>128	>128	>128	>128	-
Chloramphenicol	32	128	>128	8	8	>128
Rifampicin	0.125	64	128	<0.0625	<1	16

3.4. Biofilm Activity Results

Enhanced biofilm formation was observed when the two isolates of *S. pseudintermedius* were grown in TSA supplemented with 1% glucose. Minimal inhibitory concentrations of antibiotics against bacterial biofilm are listed in Table 1. Isolate one biofilm was resistant to all antibiotics except for ceftiofur and moxifloxacin with MICs <0.25 and 8 µg/ml respectively. But the minimum biofilm eradication concentration (MBEC) of this isolate was susceptible with ceftiofur only with 2 µg/ml concentration. Isolate two biofilm was resistant to all antibiotics except for tetracycline, amikacin, doxycycline, chloramphenicol and rifampicin with MICs <0.125 and 8 µg/ml, respectively. Meanwhile, the minimum biofilm eradication concentration (MBEC) of this isolate was susceptible with rifampicin only with 16 µg/ml concentration.

3.5. Treatment Results

Case one was treated with topical cefazolin q6h was instituted and the conjunctivitis resolved. However long-term topical therapy with tobramycin ophthalmic solution q12h was instituted in an effort to prevent future recurrence of the bacterial conjunctivitis. Meanwhile case two was treated with topical levofloxacin plus oral doxycycline for three weeks resulting in complete resolution without complication. A second culture was obtained from the case after four weeks to confirm clearance of infection.

4. Discussion

Results from this study demonstrate that the two isolates of *S. pseudintermedius* had different antibiotic susceptibilities; Isolate One was susceptible to ticarcillin, ceftiofur, cefazolin, moxifloxacin, amikacin and rifampicin with MICs ranging 0.03125–4 µg/ml. However, Isolate Two was susceptible to ceftiofur, gatifloxacin, cefazolin, moxifloxacin, tetracycline, doxycycline and rifampicin with MICs ranging <0.0625-4 µg/ml. However, both isolates were susceptible to ceftiofur, cefazolin, moxifloxacin and rifampicin. The current study agrees with the findings of Kang et al. [12], in which 4.1% of *S. pseudintermedius* isolated from canine eyes were resistant to moxifloxacin, which indicated that the majority of isolates were susceptible to moxifloxacin. Meanwhile, Ruzauskas et al. [13] reported that *S. pseudintermedius* isolates were resistant to rifampicin and tetracycline, which disagrees with the results for Isolate One and Two respectively from this study.

The two isolates from the present study were resistant to gentamicin, bacitracin, sulfamethoxazole and erythromycin with MICs ranging 64– >128 µg/ml. In addition, Isolate One showed intermediate susceptibility to trimethoprim and neomycin; at the same time, Isolate Two was resistant to both antibiotics. Previous studies reported wide ranges of resistance of *S. pseudintermedius* to other antibiotics such as penicillin G (94.1%), tetracycline (64.7%) and macrolides (68.7%). Resistance to fluoroquinolones ranged from 25.5% (gatifloxacin) to 31.4% (ciprofloxacin) [13]. Resistance also was observed by Kang et al. [12] for ciprofloxacin (40.8%), ofloxacin (38.8%), enrofloxacin (38.8%), levofloxacin (34.7%), and moxifloxacin (4.1%), which indicate the wide topical use of these antibiotics in ophthalmic diseases.

Our study observed that the two isolates of *S. pseudintermedius* showed enhanced biofilm formation. This finding coincides with the findings of Prado et al. [14] and Varges et al. [15] who found that all *S. intermedius* group strains isolated from conjunctivitis cases synthesized large amounts of biofilm either isolated from clinically normal dogs or from dogs with ulcerative keratitis. Also, the findings in our study agree with the study of Garbacz et al. [3] who were the first to document the synthesis of large amounts of biofilm by *S. pseudintermedius* strains isolated from cases of canine conjunctivitis. Biofilm formation promotes the survival of bacteria, protecting them against physicochemical factors and immune mechanisms of the host. Because of biofilm fragmentation and detachment, the bacteria spread throughout the body and colonize new sites, leading to the chronic and recurrent character of resultant infection. Bacteria that are immersed in biofilm show higher resistance to antibiotics, whose effective concentrations must be many times higher than in the case of planktonic bacteria [16].

Knowledge of the anti-biofilm activity of antibiotics is critical for the management of biofilm-related infections. In the present work, the selection of antibiotics was based on their relevance in the prevention and treatment of staphylococcal infections; older drugs such as chloramphenicol and tetracycline were also included, since older antibiotics are re-evaluated for treatment of multi-drug resistant and biofilm-based infections, due to the decrease in development of novel antimicrobials [17]. Evaluation of antibiotic activity against bacterial biofilm (Table 1) revealed high antibiotic resistance in the formed biofilms by the two isolates. Biofilm formation and MBEC were only susceptible to ceftiofur in isolate one. However, biofilm formation was resistant to all antibiotics in isolate two. The development of resistance of *S. pseudintermedius* to antibiotics prescribed for canine conjunctivitis may be attributed to several factors, including: the topical route for ocular therapy; the concentration, kinetics and elimination of drugs within the conjunctival sac; and corneal permeability and tear flow which affect the penetration of topical medications [18,19].

In the current study, differences in antibiotic susceptibility and resistance indicate that antibiotic sensitivity testing should be performed in dogs with bacterial conjunctivitis caused by *S. pseudintermedius* before an antibiotic is prescribed. However, in clinical practical veterinary medicine this is rarely done; when an animal presents with purulent discharge associated with suspect bacterial conjunctivitis, clinicians will always first treat with a broad-spectrum topical antibiotic, and only perform culture of a conjunctival swab in the event of failure of antibiotic therapy. If the bacterial culture is forming a biofilm, it is recommended to prescribe antibiotics for treatment of ocular surface diseases in canines based on the results of the antibiotic sensitivity for both bacterial culture and biofilm.

The complex biofilm structure of *S. pseudintermedius* might play a role by physically and/or chemically sequestering the antibiotic, thus delaying its penetration through the biofilm [20].

When ocular antimicrobial agents are administered for infections, consideration should be given to penetration of topically applied drugs to reach the site of action and duration of drug action [21]. One therapeutic consideration for dogs with bacterial conjunctivitis is to reduce the planktonic bacterial and biofilm mass on the ocular surface by frequent flushing of the eye with dilute betadine solution. This also helps eliminate mucopurulent discharge that blocks contact of topical antibiotics with the ocular surface. The most common route of administration for ocular therapy is topical. The concentration and elimination of drugs within the conjunctival sac and corneal permeability affect the penetration of topical medications [18]. Tear flow, space within the conjunctival fornix, and pharmaceutical characteristics of the drugs also influence efficacy of topical ophthalmic medications [19]. Because there are no separate standards for guiding topical antibiotic ocular therapy, the frequency of resistant bacteria appears to be considerable [10,22].

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