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Identification and antimicrobial susceptibility of microorganisms isolated from severe corneal ulcers of dogs in Thailand

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ABSTRACT. This study aims to determine the microbiological profile and risk factors associated with antimicrobial-resistant bacteria in canine severe corneal ulcers. Thirty-two corneal and conjunctival swabs were collected from dogs with diagnosed severe corneal ulcers that presented to Prasu-Arthorn veterinary teaching hospital in Nakhon Pathom, Thailand from June 2015 to June 2016. Microorganisms were identified by means of genotypic and phenotypic approaches. Of 32 ulcers sampled, 26 (81.3%) yielded culturable microorganisms with 24 bacterial isolates and 7 fungal isolates. The most commonly isolated bacteria were *Staphylococcus* spp. (45.8%, 11/24) and *Pseudomonas aeruginosa* (20.8%, 5/24). Out of 11 staphylococcal isolates identified, 10 carried the *mec*A gene providing methicillin resistance. The extended-spectrum β-lactamase (ESBL) encoding genes $bla_{CTX\text{-}M}$ and $bla_{VEB\text{-}1}$ were found in *an Acinetobacter lwoffii* isolate, and bla_{SHV} was found in a *P. aeruginosa* isolate. Based on the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint criteria, minimum inhibitory concentrations values showed that all bacteria, except for staphylococci, were susceptible to current ophthalmic antibiotics. More than 50% of staphylococci were resistant to all generations of fluoroquinolones and fusidic acid. Chloramphenicol was highly active against staphylococci (81.3% susceptible). The width (P=0.02) and the depth (P=0.04) of ulcers predicted greater risk of yielding resistant bacteria. The identification of antimicrobial-resistant bacteria prompts practitioners to be prudent when choosing ophthalmic antibiotics for severe corneal ulcers.

KEY WORDS: antimicrobial susceptibility, canine severe corneal ulcer, ophthalmic antibiotic, risk factor

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Severe corneal ulcer is a devastating ocular pathology that can lead to descemetocele formation or corneal perforation. This pathology comprises deep stromal ulcers and malacic ulcers, both of which are ophthalmic emergencies [3, 25]. The etiologies of severe corneal ulcers can be either infectious or noninfectious. The noninfectious causes of corneal ulcers include physical and chemical traumas, while the infectious causes are mostly associated with bacterial infections and, to a lesser extent, viral and fungal infections [3]. Regardless of the primary cause, secondary bacterial infections commonly arise as the opportunistic bacteria and readily found in conjunctival sac of the dogs. Most bacteria recovered from canine ulcerative keratitis are Gram-positive bacteria with *Staphylococcus* spp. constituting the major population followed by β-hemolytic streptococci and *Pseudomonas aeruginosa*, the latter two are typically isolated from canine malacic ulcer [22, 32, 38].

Antimicrobial resistance (AMR) is of great concern in human and veterinary medicines. In veterinary ophthalmology, there have been reports on AMR bacteria isolated from both healthy and diseased ocular samples [10, 26, 35]. As ophthalmic antibiotics compose the mainstay treatment regimen for corneal ulcers, their use is inevitably affected by the emergence of AMR. Therefore, we conducted this study to identify the microorganisms isolated from severe corneal ulcers in dogs and to characterize the antimicrobials of the bacterial isolates by phenotypic and genotypic approaches in order to elucidate the AMR in ocular samples and raise concern regarding the appropriate use of ophthalmic antibiotics based on *in vitro* studies.

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MATERIALS AND METHODS

Case selection

This study was conducted at Prasu-Arthorn veterinary teaching hospital, Faculty of Veterinary Science, Mahidol University (Salaya campus, Thailand) from June 2015 to June 2016. The dogs were enlisted in our study when their corneal lesions received a score of 3 for at least 3 of our 5 initial criteria as described in the following section. Any cases that received topical ophthalmic antibiotics in the 2 months prior to the examination were excluded from our study. All of the owners were informed and asked to sign a consent form before the specimens were collected.

Clinical data collection

The medical record and ocular examination results were collected from each case. The ocular examinations consisted of slit lamp biomicroscopy (Kowa SL-14; Kowa, Tokyo, Japan), indirect ophthalmoscopy (Keeler, Windsor, U.K.), fluorescein staining (32K supply, Eye Surgical, Thailand), and Schirmer I tear testing (Schering-Plough Animal Health Co., NJ, U.S.A.). For the affected eye, the ulcer was scored following our initial criteria modified from Ledbetter *et al*. [18]: ulcer depth relative to the corneal depth: 0=≤25%, 1=26–50%, 2=51–75%, 3=≥76%; ulcer area relative to the corneal area: 0=≤25%, 1=26–50%, 2=51–75%, =≥76%; corneal edema area: 0=≤25%, 1=26–50%, 2=51–75%, 3=≥76%; anterior chamber reaction: 0=none, 1=mild aqueous flare, 2=moderate to marked aqueous flare, 3 =hypopyon or hyphema; keratomalacia: 0=none, 1=mild, 2=moderate, 3=severe. The supporting criteria included other minor pathological changes associated with the eyes and adnexa. The neuro-ophthalmic examinations were performed in both eyes.

Microbiological sample collection and identification

The study protocol was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC number MUVS-2015-28). The lesion was evaluated before the sample was collected. Corneal and conjunctival swabs were collected before the application of any ophthalmic solution. The samples were collected by sterile rayon-tipped swabs and stored in Amies transport media (Delta lab, Rubí, Spain).

Bacterial isolation was performed by inoculating each sample on MacConkey agar and 5% sheep blood agar (Oxoid, Basingstoke, Hampshire, U.K.), and incubated at 37°C for 24 hr. The isolated colonies were initially classified based on Gram staining and, macroscopic and microscopic morphologies. The identification panel for Gram-positive bacteria was composed of a catalase test, a coagulase test, and culturing on mannitol salt agar. The species of each isolate was identified by 16S rRNA sequence analysis (Macrogen, Seoul, Korea). Standard biochemical tests and API-20E test kits (Biomérieux, La Balme Les Grottes, France) were used for Gram-negative bacteria.

For fungal isolation, the samples were submitted to the Laboratory of Veterinary Mycology, Mahidol University for fungal identification by conventional culture method.

Antimicrobial sensitivity testing

The antimicrobial minimum inhibitory concentrations (MICs) were evaluated broth microdilution methods. The antibiotics chosen were based on which antibiotics are commercially available as topical ophthalmic preparations. The tested drugs consisted of gentamicin (GEN), tobramycin (TOB), chloramphenicol (CHL), polymyxin-B (PMB), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), and fusidic acid (FUS) (Tokyo Chemical Industry Co., Tokyo, Japan). The interpretation of results was performed following the breakpoints recommended by Clinical Laboratory Standards Institute (CLSI) [6] and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [8] when available. *S. aureus* ATCC 25923 was used as quality control strain.

Detection of mecA-mediated oxacillin resistance and Extended-spectrum β-lactamases (ESBL) encoding genes

All staphylococcal isolates were tested for methicillin resistance. Methicillin-resistant staphylococci (MRS) were screened by disk diffusion method using oxacillin (OXA, 1 μg) on Mueller-Hinton agar (MHA) [40]. For all oxacillin-resistant isolates, *mec*A gene was confirmed by multiplex polymerase chain reaction (PCR) assay [29]. The primers for detection of *mecA* and 16S rRNA gene were used, giving products of 537 and 886 bp, respectively. The primer sequences were as follow (5′→3′): *mec*A F, GGG ATC ATA GCG TCA TTA TTC; *mec*A R, AAC GAT TGT GAC ACG ATA GCC; 16S rRNA F, GTG CCA GCA GCC GCG GTA A; 16S rRNA R, AGA CCC GGG AAC GTA TTC AC. *S.aureus* ATCC 43300 (MRSA) and 25923 were used as a positive and negative control for *mec*A gene, respectively. All Gram-negative isolates were initially screened by phenotypic confirmatory tests, which were performed on MHA with ceftazidime (CAZ, 30 μg) and cefotaxime (CTX, 30 μg) with and without clavulanic acid (CLA, 10 μg) (BD Diagnostics, Sparks, NV, U.S.A.) as described in CLSI guidelines [6]. The conventional PCR method was used to identify ESBLs encoding genes. The target genes in our study covered Bush-Jacoby functional group 1 and 2 and Ambler molecular class A, C and D [2, 4]. The selection of target genes was based on the genes that confer the resistance to antibiotics commonly used in veterinary medicine. Our targeted genes included bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, bla_{GES}, bla_{PER}, $bla_{\mathrm{VEB\text{-}1}}$(class A), bla_{CMY} (class C) and bla_{OXA} (class D). The primers for detection of bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, bla_{GES}, bla_{PER}, $bla_{\mathrm{VEB\text{-}1}}$, bla_{CMY}, and bla_{OXA} were used, giving products of 867, 593, 867, 827, 827, 643, 1243, and 885 bp, respectively [15, 20, 23].

Statistical analyses

All variables were categorized binomially, e.g., the lesions with the score of 3 were listed as severe versus the score of 0–2 as

Table 1. Isolation of microorganisms and clinical characteristics of corneal ulcers in dogs

Clinical factor	No. (%) of dogs with positive bacterial cultures (n=19)	No. (%) of dogs with positive fungal cultures (n=7)
Affected eye		
OD (right eye)	13 (68.4)	4 (57.1)
OS (left eye)	6 (31.6)	3 (42.9)
Ulcer depth >50%	18 (94.7)	4 (57.1)
Ulcer area >50%	12 (63.2)	4 (57.1)
Corneal edema >50%	15 (79.0)	5 (71.4)
Keratomalacia	14 (73.7)	5 (71.4)
Severity grade		
Mild aqueous flare	1 (5.3)	0
Moderate to marked aqueous flare	5 (26.3)	3 (42.9)
Hypopyon or hyphema	12 (63.2)	4 (57.1)
Keratomalacia with deep ulcer	8 (42.1)	2 (28.6)

non-severe. Univariate logistic regression was used to determine the influence of each variable on the cultured microorganisms. All variables with P-value ≤0.2 were further included in multivariable logistic regression model with backward elimination method. All statistical analyses were conducted using IBM SPSS Statistics 24 with the alpha set to 0.05 and CI at 95%.

RESULTS

Patient and ulcer characteristics

There were 32 cases enlisted in our study, 27 of them (84.3%) were purebred, and Shih-Tzu (8/32, 25%) and poodle (7/32, 21.9%) presented as the major populations. Of 32 dogs, 16 (50%) were brachycephalic breeds and the rest were mesencephalic breeds. There were 17 (53.1%) male dogs. The age of the dogs in this study ranged from 1.5 to 12.3 years old (data not shown). The lesion characteristics of the enlisted cases based on our 5 initial criteria are summarized in Table 1.

Microorganism identification

There were 26 samples that yielded culturable microorganisms, including 19 bacterial samples and 7 fungal samples. Those 19 bacterial samples gave 24 culturable bacterial isolates, 5 of which were positive for 2 different isolates. The bacteria isolates consisted of 11 *Staphylococcus* spp. (45.8%), 5 *Pseudomonas aeruginosa* (20.8%), 2 *Streptococcus* spp. (8.3%), 1 *Micrococcus lactis* (4.2%), 1 *Escherichia coli* (4.2%), 1 *Serratia marcescens* (4.2%), 1 *Enterobacter cloacae* (4.2%), 1 *Aeromonas* spp. (4.2%), and 1 *Acinetobacter lwoffii* (4.2%). Eleven *Staphylococcus* isolates consisted of 5 *S. pseudintermedius*, 2 *S. sciuri*, 1 *S. warneri*, 1 *S. cohnii*, 1 *S. saprophyticus,* and 1 *S. epidermidis*. Among fungal samples, 5 of them yielded pure culture of *Candida spp.*, while the rest were dematiaceous fungi.

Identification of Methicillin-resistant staphylococci and ESBLs-producers

In our study, the MRS was defined as any staphylococcal isolate that was OXA-resistant in the disk diffusion screening test and positive for *mecA* gene by PCR detection. There were 10 isolates of staphylococci (10/11, 90.9%) identified as MRS. There were 2 isolates defined as ESBL producers, an isolate of *P. aeruginosa* (1/5, 20%), which was positive for SHV-type ESBL gene, and an isolate of *A. lwoffii*, which was positive for CTX-M and VEB-1 type-ESBLs genes.

Antimicrobial sensitivity testing by broth microdilution

The MIC range and resistant isolates are summarized in Table 2. In staphylococci isolates, 6 out of the 11 isolates (54.6%) were resistant to CIP, LVX and MXF by CLSI and EUCAST. The numbers of resistant isolates to GEN, TOB and CHL by CLSI were lower than EUCAST (27.3 vs 81.8% for GEN, 27.3 vs. 90.9% for TOB and 18.2 vs. 36.4% for CHL). The breakpoint for FUS was only available from EUCAST, and 8 isolates (72.7%) were identified as resistant. All *P. aeruginosa* were susceptible to GEN, TOB, POL, CIP and LVX. In Enterobacteriaceae isolates, one *Enterobacter cloacae* was resistant to MXF and one *Serratia marcescens* was resistant to CHL by EUCAST.

Risk factors for isolated microorganisms

The univariable logistic regression is shown in Table 3. All 4 variables with P-value ≤0.2 were further analyzed with multivariable logistic regression. The ulcer area and ulcer depth (P=0.04) was significantly associated with carriage of AMR bacteria isolates. No statistically significant relationship was identified with fungal isolates.

Table 2. MIC range and percentage of resistant isolates (based on CLSI and EUCAST breakpoint) to selected ophthalmic antibiotics

Drug	*Staphylococcus* spp. (*n*=11)			*Streptococcus* spp. (*n*=2)			*P. aeruginosa* (*n*=5)			*Enterobacteriaciae* (*n*=3)			*Aeromonas* spp. (*n*=1)			*A. lwoffii* (*n*=1)		
	MIC range (*µ*g/m*l*)	% Resistant		MIC range (*µ*g/m*l*)	% Resistant		MIC range (*µ*g/m*l*)	% Resistant		MIC range (*µ*g/m*l*)	% Resistant		MIC range (*µ*g/m*l*)	% Resistant		MIC range (*µ*g/m*l*)	% Resistant	
		CLSI	EUCAST		CLSI	EUCAST		CLSI	EUCAST		CLSI	EUCAST		CLSI	EUCAST		CLSI	EUCAST
Gentamicin	≤0.5–16	27.3	81.8	≤0.5–4	NA	NA	≤0.5–2	0	0	≤0.5	0	0	≤0.5	NA	NA	1	0	0
Tobramycin	≤0.5–16	27.3	90.9	2–8	NA	NA	≤0.5	0	0	≤0.5–4	0	0	≤0.5	NA	NA	1	0	0
Chloramphenicol	4–64	18.2	36.4	2–4	0	0	64	NA	NA	8–16	0	33.3[a]	≤0.5	NA	NA	1	NA	NA
Polymyxin-B	8–≥256	NA[c]	NA	4–32	NA	NA	2	0	NA	≤0.5–≥256	NA	NA	2	NA	NA	1	0	NA
Ciprofloxacin	≤0.5–64	54.6	54.6	≤0.5	NA	NA	≤0.5	0	0	≤0.5	0	0	≤0.5	NA	NA	1	0	0
Levofloxacin	≤0.5–16	54.6	54.6	≤0.5	NA	0	≤0.5	0	0	≤0.5	0	0	≤0.5	NA	NA	1	0	0
Moxifloxacin	≤0.5–4	54.6	54.6	≤0.5	NA	NA	1–2	NA	NA	≤0.5–1	NA	33.3[b]	≤0.5	NA	NA	≤0.5	NA	NA
Fusidic acid	≤0.5–≥256	NA	72.7	8–64	NA	NA	≥256	NA	NA	≥256	NA	NA	≥256	NA	NA	≥256	NA	NA

a) *Enterobacter cloacae* isolate was resistant to moxifloxacin. b) *Serratia marcescens* isolate was resistant to chloramphenicol. c) NA, not applicable.

Table 3. Risk factors associated with antimicrobial-resistant bacteria in severe corneal ulcers of dogs

Variables	AMR bacteria		Odds ratio (95% confidence interval)	*P* value	Fungi		Odds ratio (95% confidence interval)	*P* value
	+	−			+	−		
Sex			3.71 (0.82–16.84)	0.09			1.7 (0.31–9.22)	0.54
Male	4	13			3	14		
Female	8	7			4	11		
Age group			0.71 (0.17–3.03)	0.65			1.7 (0.31–9.22)	0.54
1–10 years old	7	10			3	14		
<1 or >10 years old	5	10			4	11		
Ulcer area			9 (1.14–57.12)	0.02*			1.27 (0.79–8.3)	0.81
0–75% corneal area	6	18			5	19		
>75% corneal area	6	2			2	6		
Ulcer depth			6.11 (1.06–35.35)	0.04*			0.89 (0.76–4.85)	0.9
0–75% corneal depth	2	11			3	10		
>75% corneal depth	10	9			4	15		
Corneal edema			1.64 (0.37–7.25)	0.52			0.89 (0.16–4.85)	0.9
0–75% cornea	4	9			3	10		
>75% cornea	8	11			4	15		
Keratomalacia			0.47 (0.08–2.81)	0.41			3 (0.5–17.95)	0.23
Not present/Not severe	10	6			4	20		
Severe keratomalacia	2	2			3	5		
Anterior chamber reaction			5 (0.87–28.86)	0.07			0.75 (0.14–4.13)	0.74
Not present/Aqueous flare	2	14			3	9		
Hypopyon/Hyphema	10	11			4	16		

Asterisks (*) indicate statistically significant.

DISCUSSION

In our study, the primary bacterial population identified was *Staphylococcus* spp., which is consistent with previous studies in the United States, Brazil, Taiwan, and China [22, 32, 36, 38]. Gram-negative isolates were composed of *P. aeruginosa* and 3 members of the *Enterobacteriaceae* family, constituted 32.2% of all isolated microorganisms. *P. aeruginosa* alone constituted 50% of all Gram-negative isolates or 20.8% of all bacterial isolates, higher rate than in the previous report [10].

Our study found that AMR was relatively low among Gram-negative isolates. There were 2 isolates identified as carriers of ESBL-genes, an isolate of *P. aeruginosa* with bla_{SHV} gene and an isolate of *A. lwoffi* with $bla_{\text{CTX-M}}$ and $bla_{\text{VEB-1}}$ genes. These families of ESBLs-genes belong to molecular class A or functional group 2, the largest group of β-lactamases. This is the first report of the detection of ESBLs encoding genes among the veterinary clinical samples in Thailand. Interestingly, all of the ESBLs encoding genes detected in our study were previously reported in human medicine in Thailand [5, 9, 13].

The origin of these AMR bacteria isolated from severe corneal ulcer remains inconclusive. The major resistance pattern observed among Gram-negative and Gram-positive isolates in our study was against β-lactam antibiotics which are not commonly available as ophthalmic preparations in Thailand, suggesting external sources. This speculation is supported by several studies that suggest the bacteria involved in ocular diseases may come from extraocular origins [24, 35, 37], since the prevalence of AMR in healthy conjunctival sac is reported to be as low as 1.6% [26].

Keratomycosis or fungal keratitis is commonly encountered in human medicine among patients with compromised cornea [14]. The studies on canine keratomycosis are rare, mostly consisting of sporadic case reports on patients who are either old or immunocompromised. In our study, the samples that yielded fungi ranged from the dogs from younger than 12 months old to older than 10 years old. However, the difference among age groups was not statistically significant and the chance of yielding fungal culture was not related to the severity of the lesions. In our study, *Candida* spp. constituted the major fungal isolates (5/8, 62.5%). Interestingly, in veterinary medicine, there is only a report of *Candida* spp. keratomycosis [27]. There are more reports on other yeast species, namely, *Malassezia pachydermatis* associated with corneal ulcers [19, 30, 31]. We cannot conclude whether our findings reflect a higher incidence of these genera and their roles in severe corneal ulcer, as there is no established prevalence available.

The MIC values were interpreted with the presumption that the concentration of antibiotics in ocular tissue is higher or equal to the serum concentration [16, 28]. In this study, we determine MIC values for ophthalmic antibiotics using breakpoints from CLSI and EUCAST. Most antibiotic susceptibility rates did not change. However, we found that the numbers of resistant Staphylococci isolates were higher when determined by EUCAST breakpoints with GEN and TOB. The impact of different CLSI and EUCAST interpretive breakpoint has been previously reported [12] and serve as an example to interpret resistant results with caution, as the variation between guideline settings need to be compatible. All isolates of *P. aeruginosa*, members of *Enterobacteriaceae*, *Streptococcus* spp., and *A. lwoffii* were susceptible to all aminoglycosides and second and third generation fluoroquinolones (FQs) antibiotics as defined by CLSI and EUCAST breakpoints. This finding is also consistent with the result obtained in previous studies [10, 22]. Interestingly, high percentages of resistance were observed among staphylococcal isolates which exerted resistance against all tested antibiotics, including FQs and FUS, which are the 2 major antibiotics used in cases of severe corneal ulcers in Thailand. FUS is a narrow spectrum antibiotic primarily effective against Gram-positive bacteria including MRS [7, 39]. Nevertheless, we found 8 MRSs that were resistant to FUS. Additionally, there are reports on the MRS that are also resistant to FQs [1, 21]. Moreover, a study in Korea found that *S. pseudintermedius* with *mecA* is approximately twice as resistant to FQs as the strain without *mecA* [11].

According to our studies, chloramphenicol (CHL) showed the least resistance among all isolates, including staphylococci. This finding is may be observed because CHL is not commonly used in Thailand, either systemically or topically. Nevertheless, the drug potency should be considered together with the ability to penetrate and retain its concentration in the cornea and aqueous humor for the best clinical efficacy. To this effect, fourth generation FQs appear to have superior efficacy [34]. As our study provided only the drug potency from *in vitro* studies, it may not truly represent the clinical efficacy. Moreover, the toxicity should also be considered, making CHL the less desirable choice [35].

The higher the severity of the lesions is, greater the risk of yielding AMR bacteria is, particularly with a wide area ulcer. An association between AMR bacterial isolates and increased severity of corneal ulcers has been reported, e.g., MRSA keratitis in dogs [35] and horses [17]. Although the reasons for this association warrant further investigation, there is evidence that the capacity of corneal cells to be invaded is related with invasive strains of bacteria, especially for *S. aureus* and *P. aeruginosa*. Interestingly, high rates of AMR bacteria were invasive strains [33].

In conclusions, antibiotics are the mainstay of the treatment regimen for corneal ulcers. The emergence of AMR bacteria poses a threat to the successful resolution. Wide and deep ulcers are associated with a greater chance of yielding AMR bacteria. Bacteria are the major microorganisms isolated from canine severe corneal ulcers but fungi are also present in notable numbers. Staphylococci constituted the major bacterial population isolated in our study. These bacteria also possess the multidrug and extensively drug-resistant characteristics. *mecA* and *bla* genes were detected in Gram-positive and Gram-negative bacteria in our study. According to our *in vitro* study, chloramphenicol remains the most effective antibiotic against MDR and XDR staphylococci. However, the true clinical efficacy of ophthalmic antibiotics depends on their potency coupled with their pharmacokinetics which merits further investigation.

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