Original Article

Investigation of Extended-Spectrum Beta-Lactamase (ESBL)-producing Escherichia coli and antimicrobial resistance in dogs with periodontal disease

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Abstract

Oral and faecal samples were collected from thirty four dogs with periodontal disease in Chiang Mai, Thailand to investigate the prevalence of Extended Spectrum Beta Lactamase -producing *E. coli* (ESBL-EC) and to determine the antimicrobial resistance patterns of isolates against 12 antimicrobial agents. Ten of thirty-four dogs (29.41%) were positive for ESBL-EC, one dog (2.94%) was positive from an oral sample and nine dogs (26.47%) were positive from faecal samples. All ESBL-EC isolates were multi-drug resistant (i.e. against ≥ 3 antimicrobial classes). All isolates were 100% resistant to Ampicillin, Cefazolin, Cephalexin, Ciprofloxacin and Clindamycin, and 100% susceptible to Imipenem. AMP-AMC-KZ-CL-ATM-DO-CIP-SXT-DA was the most common resistance pattern identified.

Keywords: *shcherichia coli*, Extended spectrum β-lactamases, Periodontal disease, Dog

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Introduction

Periodontal disease (PD) is characterized by localized bacterial infections and an inflammatory response that directly affects one or more of the periodontal tissues, such as the alveolar bone, periodontal ligaments, cementum and gingiva (Kamel *et al.*, 2007; Kaur *et al.*, 2017).Periodontal disease is multifactorial, caused primarily by dental plaque microorganisms with predisposing factors including environment, chewing behavior, nutrition, oral hygiene, genetics (Albuquerque *et al.*, 2012) and systemic health status, for example renal, hepatic or cardiac disorders or immune-mediated disease (Glickman *et al.*, 2009; Pavlica *et al.*, 2008). In many surveys PD was been found in approximately 80% of dogs and 70% of cats aged 3 years or older (Harvey, 1998). The prevalence increases with age and small or toy breeds are particularly susceptible (Harvey *et al.*, 1994; Kortegaard *et al.*, 2008). Over 500 different species of bacteria have been isolated from dental plaque. The bacterial flora of clinically healthy gingiva is mainly composed of aerobic and facultative anaerobic Gram-positive species (Crossley *et al.*, 1995). With supra-gingival plaque accumulation, gingivitis develops and the bacteria shift from Gram-positive to Gram-negative organisms (Marsh, 1994).

Although *E. coli* is not a predominant species in oral biofilms it is commonly found in the gastrointestinal tract of both humans and animals and can be transmitted via the faecal-oral route (Centers for Disease Control and Prevention, 2015). There have been many reports of ESBL-EC from the gastrointestinal tract of both sick and healthy dogs, with a worldwide prevalence of up to 40% (Albrechtova *et al*., 2014; Carattoli *et al*., 2005; Ewers *et al*., 2010; Huber *et al*., 2013; Moreno *et al*., 2008; Keefe *et al*., 2010; Pasotto *et al*., 2016; Shaheen *et al*., 2011; Sun *et al*., 2010; Tamang *et al*., 2012; Wagner *et al*., 2014). In a previous study, Oliveira *et al.* (2016) detected *E. coli* in the oral cavity of healthy dogs and demonstrated that these strains carried the extended spectrum beta-lactamase (ESBL) enzyme. These findings indicate that dogs may contribute to the dissemination of resistant bacteria to other animals and humans.

The present study was designed to investigate the presence of ESBL-EC in dogs with periodontal disease and to determine the antimicrobial resistance patterns of any strains that were isolated.

Materials and Methods

Study population: Oral biofilm and faecal samples were obtained from 34 dogs presented to the dental clinic of the Veterinary Teaching Hospital, Chiang Mai University, from November 2015 - November 2016 with stage 2 periodontal disease, classified as early periodontitis, with 25% or less of alveolar bone loss (American Veterinary Dental College, 2009). This number was estimated with an expected prevalence of 10%, with a precision of 5% and 95% confidence. Dogs showing signs of gingivitis and periodontitis were included in the study and samples were collected by purposive sampling of any age, breed and sex. All dog owners gave written informed consent and completed a questionnaire before enrollment in this study.

Questionnaire: Data were collected including signalment, vaccination and deworming histories, antimicrobial usage in the previous 3 months, sterilization, diet, faecal appearance and present illness. The protocol for this study was approved by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand. (Ref.No. R6/2558)

Sample collection and Escherichia coli isolation: Faecal and oral biofilms were collected from each dog by swab using a sterile technique. The faecal samples were collected per rectum using a sterile cotton swab and immediately immersed into 9 ml of Luria Bertani broth at room temperature. Oral biofilm samples were collected using a sterile cotton swab from both buccal and palatal sites of every tooth and immediately immersed into 9 ml of Luria Bertani broth at room temperature. Samples were transported to the microbiology laboratory within 24 h. Both faecal and oral samples were streaked onto MacConkey agar and the MacConkey agar was supplemented with 1µg/mL cefotaxime plates and incubated for 24 h at 37 °C following a protocol described by *Rocha-Gracia et al* (2015) to isolate colonies resistant to cefotaxime and cephalosporin . Two typical *E. coli* colonies were tested to confirm their identity using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Boonyasiri *et al.*, 2014; Carbonnelle *et al.*, 2011; Gongora *et al*., 2015).

Extended-spectrum β-lactamase (ESBL) phenotype identification: Two typical CTX^r *E. coli* colonies per individual sample from MacConkey agar plus 1µg/mL cefotaxime plates were examined for extended-spectrum β-lactamase (ESBL) production by the combination disk test. This involved streaking colonies on Mueller Hinton agar plates containing cefotaxime (CTX, 30µg) and ceftazidime (CAZ, 30µg) alone and in combination with clavulanic acid (CAL) (CTX 30 µg + CAL 10µg, CAZ 30µg + CAL 10µg) (Clinical and Laboratory Standards Institute, 2014). The inhibition zone around the cephalosporin disc combined with clavulanic acid was compared with the zone around the disc with the cephalosporin alone. The test was positive if the inhibition zone diameter was ≥ 5 mm with clavulanic acid rather than without.

Antibiotic susceptibility testing: Antimicrobial susceptibility testing was performed by disc diffusion method for ampicillin (AMP, 10µg), amoxicillin-clavulanic acid (AMC, 30µg), cefazolin (KZ, 30 µg), cephalexin (CL, 30 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), gentamicin (CN, 10 µg), doxycycline (DO, 30 µg), norfloxacin (NOR, 10 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), and clindamycin (DA, 2 µg) following Clinical and Laboratory Standards Institute M100-S24guidelines (Clinical and Laboratory Standards Institute, 2014). Isolates were classified as susceptibile or resistant according to the guidelines. Intermediate isolates were reported as susceptible. *E .coli* ATCC 25922 was used as a quality control strain.

Statistical analysis: R program version 3.3.2 was used to identify antimicrobial drug resistance patterns. Independent variables were created from the questionnaires. Logistic regression and Fisher's exact test were used to examine the association between variables and all outcomes. A significant level is $P \leq 0.05$.

Results

Phenotypic detection of ESBL: CTX^R positive *E. coli* isolates were detected in 10/34 (29.41%) dogs with periodontal disease. All the CTX^R positive *E. coli* isolates exhibited an ESBL phenotype. Of the 10 dogs that produced ESBL enzymes, one dog (2.94%) was positive from an oral biofilm sample and nine different dogs (26.47%) were positive from faecal samples. All 10 dogs had both gingivitis and periodontitis and were treated by dental scaling and polishing. The median age of the dogs was 5 years (range: 3-13 y). The most common sex of the dogs was male (n = 6/10) .The most common breed of dog was poodle (n= 5/10), the other breeds were mixed breed, shih tzu and mini bull terrier (n = 3, 1 and 1 respectively).

Antimicrobial resistance: Results of agar disk diffusion indicated that all 20 ESBL *E. coli* isolates (two isolates per individual dog) were resistant to AMP, KZ, CL, CIP and DA (20 isolates, 100%), followed by ATM (18 isolates, 90%), DO and SXT (12 isolates, 60%), CN and NOR (8 isolates, 40%) and AMC (6 isolates, 30%). In contrast, IMP was susceptible to all isolates. Nine antimicrobial resistance patterns were identified from the 20 *E. coli* isolates and all could be classified as multidrug resistant (MDR) (Table1). The largest number of antimicrobial drug resistant was 11 drugs, while the least of antimicrobial drug resistant were 6 drugs. The same isolates from individuals exhibited the same antimicrobial resistance patterns.

Table 1 Antimicrobial resistance patterns of ESBL producing *E.coli* isolates using agar disk diffusion method.

	Isolate source	Antimicrobial resistance pattern
Periodontal disease	Dog P1 Biofilm 1	AMP-KZ-CL-ATM-CN-DO-NOR-CIP-DA
	Dog P1 Biofilm 2	AMP-KZ-CL-ATM-CN-DO-NOR-CIP-DA
	Dog P2 Feces 1	AMP-AMC-KZ-CL-ATM-CN-DO-NOR-CIP-SXT-DA
	Dog P2 feces 2	AMP-AMC-KZ-CL-ATM-CN-DO-NOR-CIP-SXT-DA
	Dog P3 feces 1	AMP-AMC-KZ-CL-ATM-DO-CIP-SXT-DA
	Dog P3 feces 2	AMP-AMC-KZ-CL-ATM-DO-CIP-SXT-DA
	Dog P4 feces 1	AMP-AMC-KZ-CL-ATM-DO-CIP-SXT-DA
	Dog P4 feces 2	AMP-AMC-KZ-CL-ATM-DO-CIP-SXT-DA
	Dog P5 feces 1	AMP-KZ-CL-ATM-CN-NOR-CIP-DA
	Dog P5 feces 2	AMP-KZ-CL-ATM-CN-NOR-CIP-DA
	Dog P6 feces 1	AMP-KZ-CL-ATM-NOR-CIP-SXT-DA
	Dog P6 feces 2	AMP-KZ-CL-ATM-NOR-CIP-SXT-DA
	Dog P7 feces 1	AMP-KZ-CL-CN-DO-CIP-SXT-DA
	Dog P7 feces 2	AMP-KZ-CL-CN-DO-CIP-SXT-DA
	Dog P8 feces 1	AMP-KZ-CL-ATM-CIP-SXT-DA
	Dog P8 feces 2	AMP-KZ-CL-ATM-CIP-SXT-DA
	Dog P9 feces 1	AMP-KZ-CL-ATM-DO-CIP-DA
	Dog P9 feces 2	AMP-KZ-CL-ATM-DO-CIP-DA
	Dog P10 feces 1	AMP-KZ-CL-ATM-CIP-DA
	Dog P10 feces 2	AMP-KZ-CL-ATM-CIP-DA

AMP,ampicillin; AMC, amoxicillin-clavulanic acid; KZ, cefazolin; CL, cephalexin; ATM, azetreonam; IPM, imipenem; CN, gentamicin; DO, doxycycline; NOR, norfloxacin; CIP, ciprofloxacin; SXT, sulfamethoxazole trimethoprim; DA, clindamycin.

Logistical regression; ESBL producing E. coli with questionnaire data: The questionnaires from dog owners were divided into two parts; general questions and questions for periodontal disease. Logistical regression in both univariable and multivariable models found no relationship between variables and ESBL production; all variables found $P > 0.05$ (Table 2).

Discussion

To our knowledge, this is the first detection of ESBL producing *E. coli* from canine oral plaque samples in Thailand. Our study found a high prevalence of ESBL producing *E .coli* in dogs with periodontal disease, 10/43(29.41%). These strains had not been recovered from the oral cavity of humans with periodontal disease (Søraas *et al.*, 2014). Previously, ESBL producing *E. coli* had been recovered in Thailand from infected dogs (9.89%) (Hanhaboon *et al.*, 2015), cows with mastitis (6.5%) (Hinthong *et al.*, 2017) and piglets with diarrhea (12.2%) (Kramomtong *et al.*, 2008). High prevalences of ESBL-producing *E. coli* have been recorded from feces of healthy rural Thai people, ranging from 29.3% to 76.2% (Luvsansharav *et al.* 2012). ESBL producing *E. coli* have been recovered from healthy dogs in many parts of the world, including Angola (75%) (Albrechtova *et al.*, 2014), Tunisia (16.3%) (Sallem *et al.*, 2013), Portugal (15%) (Belas *et al.*, 2014), Mexico (6%) (Gracia *et al.*, 2015) and the United Kingdom (1.36%) (Schmidt *et al.*, 2015). Subsequently, in sick dogs was found 5.6% in Germany (Ewers *et al.*, 2010), 1.91% in Korea (Tamang *et al.*, 2012), 3% United states (Shaheen *et al.*, 2011) and 3.74% Switzerland (Huber *et al.*, 2013). ESBL producing *E. coli* have also been recovered from environmental samples, including water, fresh meat and vegetables (Hanhaboon *et al.*, 2015; Singh *et al.*, 2017).

In this study, ESBL producing *E. coli* was not found in both faecal and oral biofilm samples from an individual dog. This does not support the idea of intra-species transmission. Contrastingly, a previous study found inter-species and intra-species ESBL producing

E. coli transmission from the same household environment (Leite-Martins *et al.*, 2015). There is the suggestion that oral plaque ESBL producing *E. coli* in our results was received from environments such as water and soil because of licking behavior (Singh *et al.*, 2017).

In this study we detected ESBL producing *E. coli* from the oral biofilm sample from one dog. *Escherichia coli* is not a common microorganism in oral biofilms. Normally, plaque is a community of cooperating microorganisms on the teeth surface, including Gram-positive species (e.g. *Actinomyces* spp., *Streptococcus* spp.) and Gram-negative, motile, anaerobic bacteria (e.g. *Porphyromonas* spp., *Actinomyces* spp., *Neisseria* spp.) (Albuquerque *et al.*, 2012; Elliott *et al.*, 2005; Kasempimolporn *et al.*, 2003; Misirligil *et al.*, 1990). The oral infection with ESBL-producing E. coli could have occurred as a result of environmental contamination from an unknown source and the licking habits of this dog.

Table 2 Characteristics of dog with periodontal disease carrying ESBL enzyme in oral biofilm or faecal samples.

Question	AESBL N=1	BESBL N=9	P-value	
			AESBL	BESBL
General question				
Vaccination			1	0.1547
- **Yes**	1	9		
- **No**	0	0		
Deworming			1	0.0547*
- **Yes**	1	8		
- **No**	0	1		
Sterilization			1	0.3796
- **Yes**	0	4		
- **No**	1	5		
Diet			1	0.6196
- **Commercial food**	0	4		
- **Homemade food**	1	5		
Faecal appearance			1	0.9851
- **Hard**	0	3		
- **Soft stool**	1	6		
- **Watery**	0	0		
Home care			1	0.7280
- **In home**	0	0		
- **Away from home**	1	4		
- **Both in home and away from home**	0	5		
Previous illness history			1	0.4181
- **Yes**	0	4		
- **No**	1	5		
Previous antibiotic drug used			1	0.0606*
- **Yes**	0	5		
- **No**	1	4		
Question for periodontal disease				
Problem			1	0.2555
- **Dental tartar**	0	0		
- **Gingivitis/periodontitis**	1	8		
- **Dental loss**	0	0		
- **Halitalsis**	0	1		
Duration			1	0.1974
- **<1 wk**	0	1		
- **1-4 wk**	0	1		
- **>1 mo**	0	1		
- **1-3 mo**	0	2		
- **>3 mo**	1	4		
Dental floss usage			1	0.2723
- **Yes**	1	1		
- **No**	0	8		
Other illness			1	0.2639
- **Yes**	0	1		
- **No**	1	8		

AESBL= ESBL producing *E.coli* was found in oral biofilm, BESBL=ESBL producing *E.coli* was found in faecal. Markable number univaliable was found in the relationship between variables and ESBL production but the dog with a previous antibiotic drug usage and deworming a *P* value close to 0.05

All ESBL-producing *E. coli* strains isolates in this study were resistant to the aminobenzyl-penicillin group (ampicillin), cephalosporin group (cefazolin and cephalexin), fluoroquinolone group (ciprofloxacin) and lincosamide group (clindamycin) while all were susceptible to the carbapenem group (imepenem). This is similar to the results of a previous study *(Huber et al.*, 2013). The prevalence of antimicrobial resistance was higher in the dogs carrying ESBL *E. coli* than dogs carrying normal *E. coli* (Costa *et al.*, 2004). Our results

revealed resistance to common antimicrobial drugs used in both human and animal medicines and the prevalence of antimicrobial resistance (AMR) may have increased in such populations over time. This suggests that AMR is starting to attach the attention of the public. Previous studies showed that all ESBL producing *E. coli* isolated from feces of healthy or sick dogs were multidrug resistant (MDR) (Carattoli *et al.*, 2005; Moreno *et al.*, 2008; Shaheen *et al.*, 2011; Tamang *et al.*, 2012), similar to the results from our study. In addition, we found that oral biofilm isolates of ESBL producing *E. coli* were also MDR.

Normally the antibiotics recommended for the treatment of dogs with periodontal disease are amoxicillin, amoxicillin-clavulanic acid, clindamycin and metronidazole (Albuquerque *et al.*, 2012; Senhorinho *et al.*, 2012; Nelson *et al.*, 2013). These antibiotics may be effective when the biofilm contains normal microflora, however, ESBL producing *E. coli* found in the biofilm of one dog in our research was resistant to all the recommended antibiotics. This could be the result of the use of new generation cephalosporins in dogs infected with ESBL producing *E. coli* and this could lead to antimicrobial resistance development.

Results from our questionnaire differed from observations previously reported on risk factors for the carriage of ESBL producing *E. coli*. In previous studies, dogs with a history of antimicrobial therapy in the past year, dogs from shelters or breeders (Belas *et al.*, 2014) and the consumption of raw meat (Schmidt *et al.*, 2015) had a higher risk of being carriers of ESBL- producing *E. coli*, however there was no relationship to these risk factors from our questionnaire.

In conclusions, the high prevalence of ESBL producing *E. coli* in dogs with periodontal disease can develop multidrug resistance leading to the failure of antimicrobial drug use in animal. Further research could also be conducted to detect the antimicrobial resistance gene, phylogroup and molecular typing isolate in the future.

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