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## Detection of extended spectrum beta-lactam (ESBL) resistance in *Pseudomonas* species of canine origin

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### Abstract

The study aimed at the detection of extended spectrum beta-lactam (ESBL) resistance in *Pseudomonas* species isolated from healthy and diarrhoeic dogs in Andhra Pradesh (India). Rectal swabs from healthy (92) and diarrhoeic (44) dogs were subjected for isolation of *Pseudomonas* species by conventional culture methods and screened for ESBL resistance by disc diffusion method. A total of 46 *Pseudomonas* isolates were recovered (26/92 of healthy and 20/44 of diarrhoeic dogs). Phenotypic screening revealed resistance to cefotaxime (52.1%), ceftriaxone (23.9%), ceftazidime (28.2%) and aztreonam (13.0%) with an overall incidence of 58.6% (27/46) beta-lactam resistance. Overall incidence of ESBL resistant phenotype was found to be 15.2% (7/46) by combination disc method. Detection of beta-lactamase genes was carried out using a set of three multiplex PCR assays and a single uniplex PCR assay. Predominant beta-lactamase genes detected include *bla<sub>OXA</sub>* (69.5%) followed by *bla<sub>TEM</sub>* (36.9%), *bla<sub>SHV</sub>* (26.0%), *bla<sub>CTX-M</sub>* group-1 (15.2%) and *bla<sub>AmpC</sub>* (4.34%). All the isolates were found to be negative for *bla<sub>ACC</sub>*, *bla<sub>MOX</sub>* and *bla<sub>CTX-M</sub>* group-2 genes. The present study reporting the first detection of ESBL resistant phenotype in *Pseudomonas* species of canine origin in India adds to the alarming ESBL resistance reports in the gut microbiota of dogs world-wide.

**Keywords:** dogs, extended spectrum beta-lactam resistance, ESBL, *Pseudomonas*

### 1. Introduction

Beta-lactamase expression is the major resistance mechanism of bacteria to beta-lactam antibiotics, which are currently the largest family of antimicrobial drugs used in veterinary medicine [1]. Broad-spectrum beta-lactamases hydrolyze penicillins and early cephalosporins [2]. Extended spectrum beta-lactamases (ESBLs) hydrolyze penicillins, first, second and third generation cephalosporins as well as monobactams and are inhibited by beta-lactamase inhibitors [3]. AmpC beta-lactamases (ACBLs) hydrolyze penicillins, cephalosporins, cephamycins and monobactams, but are not inhibited by beta-lactamase inhibitors [4]. Based on general prevalence, ESBLs are broadly grouped into major and minor ESBLs. Major ESBLs (TEM, SHV and CTX-M) are commonly encountered in many parts of the world whereas the minor types (OXA, PER, VEB etc) are restricted to certain geographical locations only [1]. Genes encoding these beta-lactamases (*bla*) can be located on plasmids or on the chromosome [2].

Emergence of resistance in commensal gut microbiota may have an impact not only for the individual dogs concerned, but also for the human beings in contact with them [5]. Resistant gut microbiota of dogs may represent a reservoir of resistance genes for human pathogens [5]. To our knowledge, studies on ESBL antimicrobial resistance in dogs are not reported from India, till today. Hence, the present study was carried out with an objective of detection of ESBL resistance in *Pseudomonas* isolates recovered from healthy and diarrhoeic dogs in Andhra Pradesh, India.

### 2. Materials and Methods

#### 2.1 Reference strains

Beta-lactamase positive strain of *Klebsiella pneumoniae* (ATCC 700603) and beta-lactamase negative strain of *E. coli* (ATCC 25922) were used as positive controls.

#### 2.2 Collection and processing of samples

Rectal swab samples (n=136) were collected from healthy (92) and diarrhoeic (44) dogs. Good health was assumed based on defecation history i.e. no history of diarrhoea for at least 4 weeks

prior to sample collection. Isolation and identification of *Pseudomonas* species was carried out by conventional culture methods and biochemical tests [6]. Whole cell DNA was extracted by boiling and snap chilling method [7] and the absorbance of DNA was measured using Nanodrop (Thermo Scientific, USA).

### 2.3 Phenotypic screening for ESBL resistance

*Pseudomonas* isolates were subjected to antibiotic sensitivity testing by disc diffusion method on Mueller Hinton agar [8]. Inhibition zone diameters were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9, 10]. CLSI recommends two-step procedure for the phenotypic detection of ESBL production, which includes an initial 'screening test' to detect resistance against one or more indicator substrates followed by 'confirmatory test' using one or more of the indicator substrates in combination with a beta-lactamase inhibitor, looking for synergy effects. Isolates were screened for resistance against four antimicrobial agents: cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CTR, 30 µg) and aztreonam (AT, 30 µg). Resistance to at least one of the indicator antibiotics was considered as 'positive' screening test [9, 10].

### 2.4 Phenotypic confirmation of ESBL resistance

Isolates that were found positive in 'screening test' were subjected to 'confirmatory test' by combination disc method. Three pairs of discs (i.e., with and without clavulanic acid) were placed: ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg), cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg) and ceftriaxone (CTR, 30 µg), ceftriaxone plus sulbactam (CIS, 30/10 µg). An isolate was confirmed for ESBL production when the inhibition zone diameter around combination discs was  $\geq 5$  mm (synergy effect) when compared to discs containing respective cephalosporin alone [9, 10].

### 2.5 Molecular detection of beta-lactamase (*bla*) genes

For the detection of beta-lactamase genes in *Pseudomonas* species, a set of three multiplex PCR assays [11] and a single uniplex PCR [12] were standardized. The use of positive (positive DNA) and negative (nuclease free water) controls was adhered to in all the PCR assays. Oligonucleotide primers used and their respective amplicon sizes were given in Table-1. All the three multiplex PCR assays were carried out in Kyratex thermal cycler (Australia) under following standardized cycling conditions - initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, elongation at 72°C for 1 min, final elongation at 72 °C for 7 min and hold at 4°C.

#### 2.5.1 Multiplex PCR-I for the detection of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> genes

Reaction mixture was optimized in 25 µl volume containing 2 µl of DNA template prepared from each isolate; *Taq* buffer (10x) - 3 µl; dNTP mix (10mM) - 1 µl; Mg Cl<sub>2</sub> (25mM) - 1.5 µl; three forward primers (10 pmol/µl) - each 0.5 µl; three reverse primers (10 pmol/µl) - each 0.5 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water - 13.5 µl.

#### 2.5.2 Multiplex PCR-II for the detection of *bla*<sub>CTX-M</sub> group 1 and 2 genes

Reaction mixture was optimized in 25 µl volume containing 1.5 µl of DNA template prepared from each isolate; *Taq* buffer (10x) - 2.75 µl; dNTP mix (10mM) - 0.5 µl; MgCl<sub>2</sub> (25mM) - 1 µl; two forward primers (10 pmol/µl) - each 0.75 µl; two reverse primers (10 pmol/µl) - each 0.75 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water - 15.25 µl.

#### 2.5.3 Multiplex PCR-III for the detection of *bla*<sub>ACC</sub> and *bla*<sub>MOX</sub> genes

Reaction mixture was optimized in 25 µl volume containing 2 µl of DNA template prepared from each isolate; *Taq* buffer (10x) - 2.75 µl; dNTP mix (10mM) - 1 µl; MgCl<sub>2</sub> (25mM) - 1.5 µl; two forward primers (10 pmol/µl) - each 0.6 µl; two reverse primers (10 pmol/µl) - each 0.6 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water - 14.35 µl.

#### 2.5.4 Uniplex PCR assay for the detection of *bla*<sub>AmpC</sub> gene

The PCR was optimized in 25 µl reaction mixture (containing 1 µl of DNA template prepared from each isolate; *Taq* buffer [10x] - 2.5 µl; dNTP mix [10mM] - 0.5 µl; MgCl<sub>2</sub>[25mM] - 1.5 µl; forward primer [10 pmol/µl] - 1 µl; reverse primer [10 pmol/µl] - 1 µl; *Taq* DNA polymerase [1 U/µl] - 1 µl and nuclease free water - 16.5 µl) under the following standardized cycling conditions: initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. Final extension was done at 72°C for 10 min.

## 3. Results and Discussion

### 3.1 Isolation and identification

Of the 136 rectal swab samples analyzed, a total of 46 *Pseudomonas* isolates were recovered which includes 26 (28.2%) isolates from healthy dogs (n=92) and 20 (45.4%) isolates from diarrhoeic dogs (n=44). In a study from Chhattisgarh (India), *Pseudomonas* species were isolated from rectal swabs of 16% healthy dogs and 33.3% of diarrhoeic dogs examined [13]. Numerous variables have been known to play part in the variation in proportion of bacterial species isolated across various studies like genetic determinants [14], diet [15], environment [16], age [17] etc.

**Table 1:** Primers used for detection of beta-lactamase genes in *Pseudomonas* species

Primer	Primer sequence (5'-3')	Amplicon size
Multiplex PCR -I (detection of beta-lactamase genes - TEM, SHV and OXA)		
<i>bla</i> <sub>TEM</sub> gene	F: CATTTCGGTGTGCGCCCTTATTC	800 bp
	R: CGTTCATCCATAGTTGCCTGAC	
<i>bla</i> <sub>SHV</sub> gene	F: AGCCGCTTGAGCAAATTA AAC	713 bp
	R: ATCCCGCAGATAAATCACCAC	
<i>bla</i> <sub>OXA</sub> gene	F: GGCACCAGATTCAACTTTCAAG	564 bp
	R: GACCCCAAGTTTCTGTAAGTG	
Multiplex PCR -II (detection of beta-lactamase genes - CTX-M group 1 and 2)		
<i>bla</i> <sub>CTX-M</sub> group 1	F: TTAGGAAATGTGCCGCTGTA	688 bp

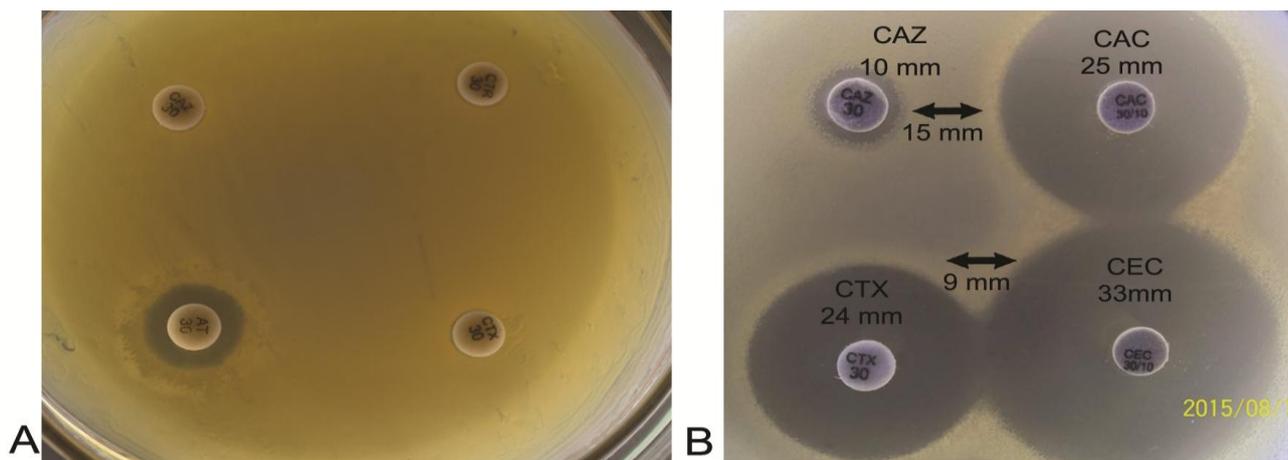
	R: CGATATCGTTGGTGGTACCAT	
<i>bla<sub>CTX-M</sub></i> group 2	F: CGTTAACGGCAGATGAC	404 bp
	R: CGATATCGTTGGTGGTACCAT	
Multiplex PCR –III (detection of beta-lactamase genes – ACC and MOX)		
<i>bla<sub>ACC</sub></i>	F: CACCTCCAGCGACTTGTAC	346 bp
	R: GTTAGCCAGCATCACGATCC	
<i>bla<sub>MOX</sub></i>	F: GCAACAACGACAATCCATCCT	895 bp
	R: GGGATAGGCGTAACTCTCCCAA	
Uniplex PCR (detection of <i>bla<sub>AmpC</sub></i> gene)		
<i>bla<sub>AmpC</sub></i>	F: CCCCCTTATAGAGCAACAA	631 bp
	R: TCAATGGTCGACTTCACACC	

### 3.2 Screening for ESBL production

As a part of first step recommended by CLSI, isolates were screened for resistance to four indicator cephalosporins. Since ESBLs vary in their hydrolysis of cephalosporins, resistance to at least one of them should be considered as positive in the screening test [9, 10]. Twenty seven out of 46 *Pseudomonas* isolates screened were found to be resistant to one or more of the screening agents, with an overall incidence of 58.6% (27/46) beta-lactam resistance and were designated as ‘suspect ESBL producers’. The ‘suspect ESBL producers’ identified include 15 (57.6%) isolates from healthy dogs and 12 (60.0%) isolates from diarrhoeic dogs. Among 26 isolates from healthy dogs, resistance to cefotaxime was observed in 13 (50.0%), ceftazidime in 8 (30.7%), ceftriaxone in 5 (19.2%) and aztreonam in 1 (3.84%) isolate. Among 20 isolates from diarrhoeic dogs, resistance to cefotaxime was observed in 11 (55.0%), ceftriaxone in 6 (30.0%), ceftazidime in 5 (25.0%) and aztreonam in 5 (25.0%) isolates. Overall incidence of resistance to cefotaxime, ceftazidime, ceftriaxone and aztreonam was found to be 52.1 (24/46), 28.2 (13/46), 23.9 (11/46) and 13.0% (6/46), respectively. In a study on antimicrobial sensitivity of 106 *Pseudomonas* species isolated from canine infections in Canada, resistance to ceftriaxone (39%), cefotaxime (26%) and ceftazidime (7%) was reported [18].

### 3.3 Confirmation of ESBL production

As a part of second step recommended by CLSI, when third generation cephalosporin discs with and without clavulanic acid/sulbactam are placed on Mueller Hinton agar inoculated with test organism, an increase in the inhibition zone diameter of  $\geq 5$  mm was observed in discs with clavulanic acid/sulbactam over the discs with cephalosporins alone, indicating ESBL production [9, 10]. Since ESBLs are inhibited by clavulanic acid/sulbactam, tests that use these inhibitors to reverse ESBL resistance were used commonly for phenotypic confirmation of ESBL production [9, 10]. Of the 27 ‘suspect ESBL producers’, ESBL production was confirmed in 7 isolates by combination disc method, which includes 2 (7.69%, 2/26) isolates from healthy dogs and 5 (25.0%, 5/20) isolates from diarrhoeic dogs. In the remaining 20 isolates, clavulanic acid synergy (5 mm principle) was not detected, which might be due to concurrent production of other non-ESBL beta-lactamases like ACBLs and metallo beta-lactamases that are resistant to beta-lactamase inhibitors, masking the 5 mm synergy in the confirmatory test [9,19,20]. The overall incidence of ESBL resistance phenotype in *Pseudomonas* species was found to be 15.2% (7/46). The observed levels of ESBL resistance phenotype among canine microbiota appeared to range from 1.4 to 41.3% in different studies i.e. 1.4% in UK [21], 4% in Pennsylvania [20], 20% in Chili [22] and 41.3% in China [23]. Mueller Hinton agar plates showing positive ‘screening’ and ‘confirmatory’ test results are shown in Figure 1.



**Fig 1:** (A). Mueller Hinton agar plate showing positive ‘screening’ test for ESBL production on *Pseudomonas* species. Cefotaxime, CTX - 0 mm (resistant); Ceftriaxone, CTR – 0 mm (resistant); Aztreonam – 12 mm (resistant) and Ceftazidime, CAZ - 0 mm (resistant). (B). Mueller Hinton agar plate showing positive ‘confirmatory’ test for ESBL production with CAZ-CAC (Ceftazidime, Ceftazidime plus clavulanic acid) and CTX-CEC (Cefotaxime, Cefotaxime plus clavulanic acid) combinations on *Pseudomonas* species.

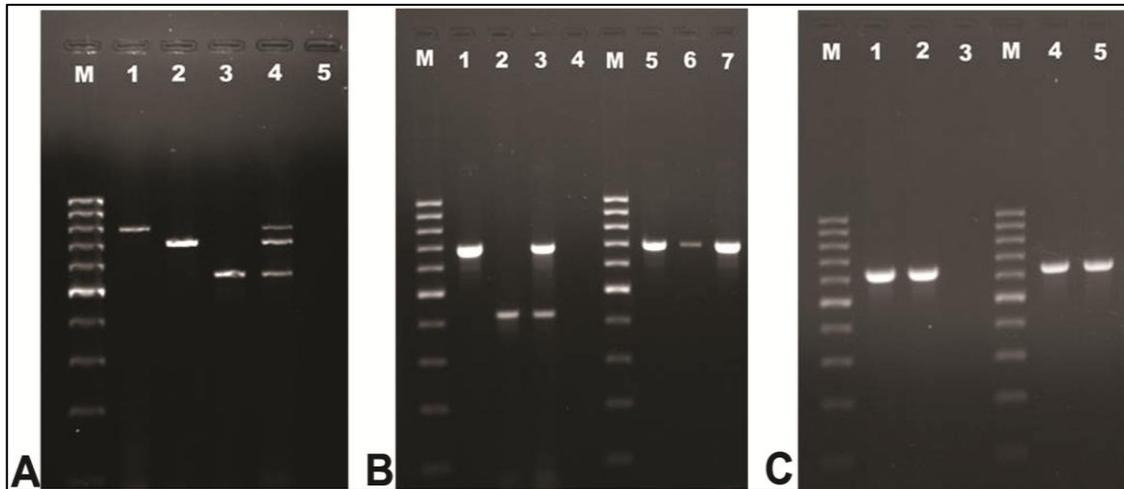
### 3.4 Detection of beta-lactamase (*bla*) genes

The overall incidence of beta-lactamase genes in *Pseudomonas* species was found to be 91.3% (42/46). Among the 26 isolates from healthy dogs, one or more beta-lactamase genes were detected in a total of 23 (88.4%) isolates whereas

3 (11.6%) isolates were negative for all the genes screened for. Beta-lactamase genes detected were as follows-*bla<sub>OXA</sub>* gene in 15 (57.6%) isolates followed by *bla<sub>TEM</sub>* gene in 12 (46.1%), *bla<sub>SHV</sub>* gene in 6 (23%), *bla<sub>CTX-M</sub>* group 1 gene in 2 (7.69%) and *bla<sub>AmpC</sub>* gene in 1 (3.84%) isolate whereas none

of the isolates harboured *bla*<sub>ACC</sub>, *bla*<sub>MOX</sub> and *bla*<sub>CTX-M</sub> group 2 genes. Among the 20 isolates from diarrhoeic dogs, one or more beta-lactamase genes were detected in a total of 19 (95%) isolates. Beta-lactamase genes detected were as follows-*bla*<sub>OXA</sub> gene in 17 (85.0%) isolates followed by *bla*<sub>SHV</sub> gene in 6 (30.0%), *bla*<sub>TEM</sub> gene in 5 (25.0%), *bla*<sub>CTX-M</sub> group 1 gene in 5 (25.0%) and *bla*<sub>AmpC</sub> gene in 1 (5.0%) isolate, whereas none of the isolates harboured *bla*<sub>ACC</sub>, *bla*<sub>MOX</sub> and *bla*<sub>CTX-M</sub> group 2 genes. Overall incidence of beta-lactamase genes *bla*<sub>OXA</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub> group 1 and *bla*<sub>AmpC</sub>

genes was found to be 69.5 (32/46), 36.9 (17/46), 26.0 (12/46), 15.2 (7/46) and 4.34% (2/46), respectively. Gel photographs of amplicons of PCR assays targeting beta-lactamase genes are shown in Figure 2. OXA type ESBLs were shown to be predominantly present in *Pseudomonas* species [24]. Incidence of beta-lactamase genes in canine microbiota was found to range from 17.5 to 82.2% across various studies i.e., 17.5% in Tunisia [25], 56.5% in UK [21] and 82.2% in the Netherlands [26].



**Fig 2:** (A). Agarose gel electrophoresis of amplicons of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> genes of *Pseudomonas* species. Lane M: 100 bp DNA ladder, L1: *bla*<sub>TEM</sub> gene (800 bp), L2: *bla*<sub>SHV</sub> gene (713 bp), L3: *bla*<sub>OXA</sub> gene (564 bp), L4: DNA standard carrying *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> genes and L5: Negative control (B). Agarose gel electrophoresis of amplicons of *bla*<sub>CTX-M</sub> group 1 and 2 genes. Lane M: 100 bp DNA ladder, L1: DNA standard for *bla*<sub>CTX-M</sub> group 1 gene (688 bp), L2: DNA standard for *bla*<sub>CTX-M</sub> group 2 gene (404 bp), L3: DNA standard carrying both *bla*<sub>CTX-M</sub> group 1 and 2 genes, L4: Negative control, L5 to L7: *bla*<sub>CTX-M</sub> group 1 gene (688 bp) of *Pseudomonas* isolate (C). Agarose gel electrophoresis of amplicons of *bla*<sub>AmpC</sub> gene of *Pseudomonas* species. Lane M: 100 bp DNA ladder, L1 and L2: DNA standard for *bla*<sub>AmpC</sub> gene (631 bp), L3: Negative control; L4 and L5: *bla*<sub>AmpC</sub> gene of *Pseudomonas* isolates.

Among the *Pseudomonas* isolates (n=7) with confirmed 'ESBL' resistant phenotype, multiple beta-lactamase genes co-existed in all the isolates. Among the isolates (n=20) that exhibited 'non-ESBL' resistant phenotype (isolates with positive screening and negative confirmatory test results), beta-lactamase genes were detected in 19 isolates whereas no beta-lactamase genes were detected in one isolate. Several explanations had been put forward by many workers for the possible expression of resistant phenotype in the absence of beta-lactamase genes [9, 26]. One explanation could be the presence of beta-lactamase genes that were not screened for or the contribution of other resistance mechanisms, such as efflux pumps [9, 26]. Among the isolates (n=19) with 'sensitive' phenotype, beta-lactamase genes were detected in 16 isolates whereas no beta-lactamase genes were detected in 3 isolates. The possible explanation for the detection of beta-lactamase genes in phenotypically sensitive isolates could be that the genes detected could be of broad spectrum beta-lactamase group [26, 27], conferring resistance to first and second generation cephalosporins but not to third generation cephalosporins used in screening test of the present study. This was evidenced in the present study, when the phenotypically sensitive isolates carrying beta-lactamase genes (n=16) were subjected to *in vitro* antibiotic sensitivity assay against first and second generation cephalosporins, remarkable percentage resistance was noticed in all the isolates.

#### 4. Conclusions

In the present study, 57.6 and 60.0% of the *Pseudomonas* isolates recovered from healthy and diarrhoeic dogs, respectively were found to possess beta-lactam resistance. In addition, ESBL phenotype was confirmed in 15.2% of the *Pseudomonas* isolates. Though, there are reports of phenotypic and genotypic detection of beta-lactam resistance in farm animals and poultry from India, studies in dogs are lacking. In this context, our present report is the first report of characterization of ESBL resistance in *Pseudomonas* species of canine origin in India.

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