

Original Research Article

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Screening Canine Microbiota for Extended Spectrum Beta-Lactamases (ESBLs) Using E-Test

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ABSTRACT

Extended spectrum beta-lactamase (ESBL) production by animal pathogens is an emerging problem world-wide. E-test was performed for the detection of ESBL production in microbiota isolated from pyometra and otitis clinical cases of dogs presented to Veterinary clinics in Andhra Pradesh. Microbiota recovered from pyometra pus samples includes *Escherichia coli* (7, 46.6%), *Staphylococcus* (11, 73.3%) and *Pseudomonas* (12, 80%) species. Microbiota recovered from otitis pus samples includes *Pseudomonas* (8, 53.3%), *Proteus vulgaris* (9, 60%) and *Staphylococcus* (15, 100%) species. Of the seven *E. coli* isolates recovered, three (43%) isolates were positive for simultaneous production of ESBL and Amp C, two (28.5%) isolates were ESBL negative and two (28.5) isolates gave non-conclusive results. Of the 20 *Pseudomonas* isolates, 16 (80%) isolates were ESBL negative and four (20%) isolates gave non-conclusive results. All the 26 *Staphylococcus* and nine *Proteus* isolates were found negative for ESBL production.

Keywords

E. coli, ESBL, Otitis, *Pseudomonas*, *Proteus*, Pyometra, *Staphylococcus*.

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Introduction

The extended spectrum beta-lactamases (ESBLs) are the bacterial enzymes that inactivate third-generation cephalosporins, penicillins and monobactams (Bush and Jacoby, 2010). ESBLs are widely detected in various human medical institutions and less frequently from the bacterial population circulating in animals (Carattoli, 2008). Various phenotypic methods used for the detection of ESBLs include combination disc method (CDM), double disc synergy test (DDST), inhibitor-potentiated disc diffusion (IPDD), three-dimensional (3D) test, E-test (Epsilon meter gradient test), commercial

automated systems like Phoenix, Vitek 2, MicroScan and Sensititre (Drieux *et al.*, 2008 and Sharif *et al.*, 2017). All these methods are based on the principle of antagonism of the action of ESBLs by beta-lactamase inhibitors like clavulanic acid (Drieux *et al.*, 2008). Epsilon meter or E-test uses thin E-strips with minimum inhibitory concentration (MIC) scale in µg/mL and two predefined antibiotic gradients (a gradient of cephalosporin alone at one end and the same gradient with a constant concentration of clavulanic acid at other end) (Drieux *et al.*, 2008). They are intended to confirm the presence of clavulanic acid

inhibitable ESBL enzymes in various bacterial species (Prabha *et al.*, 2016).

The aim of this study is to screen the microbiota isolated from clinical cases of pyometra and otitis in dogs for ESBL production using E-test method.

Materials and Methods

Sample collection

Pus swab samples from clinical cases of otitis (n=15) and pyometra (n=15) were collected from dogs presented to Teaching Veterinary Clinical Complex (TVCC) of College of Veterinary Science (Tirupati), NTR College of Veterinary Science (Gannavaram) and Veterinary Polyclinic (Ongole), Andhra Pradesh, India. All the samples were transported on ice to the laboratory immediately after collection.

Isolation and identification of microbiota

Pus swab samples were transferred to nutrient broth and incubated aerobically at 37°C for 24 hours. Enriched samples were streaked on nutrient agar and mannitol salt agar plates, incubated at 37°C and the colonies were tentatively identified based on the colonial morphology, swarming phenomenon, pigment production, Gram's staining and sub-cultured on MacConkey agar. Lactose fermenting pink colonies on MacConkey agar were further streaked onto EMB agar plates. Non-lactose fermenting pale colonies on MacConkey agar were further streaked onto XLD agar plates. Identification of the bacterial isolates to the genus/species level was done using conventional biochemical tests (Sneath and Holt, 2001).

E-test method

The determination of MIC of cefotaxime and cefotaxime/clavulanic acid for the bacterial

strains isolated in the present study was performed using E-test strips (Hi-Media, Mumbai, India) (CTX / CTX+) containing a fixed concentration gradient of cefotaxime alone (CTX, 0.25 – 16 µg/mL) at one end of the strip and cefotaxime with clavulanic acid (CTX+, 0.016 – 1.0 µg/mL) on the other end. E-test strips were applied aseptically on Mueller-Hinton agar plates inoculated with pure culture of bacterial isolates, by pressing it with sterile forceps along the whole length of the antibiotic gradient ensuring the complete contact with agar surface. Results were recorded after 18-24h of incubation at 37°C. Inhibition of growth presents as a spherical formation or inhibition ellipse. The MIC is read as the point where the inhibition ellipse intersects the edge of the E-test strip.

The presence of an ESBL was confirmed if there is an 8 fold reduction (MIC ratio of CTX and CTX+ \geq 8) in the MIC of cefotaxime in presence of clavulanic acid (i.e., MIC value of cefotaxime reduced by more than three doubling dilution steps in the presence of clavulanate) (Drieux *et al.*, 2008). *E. coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) were used as negative and positive controls, respectively.

Results and Discussion

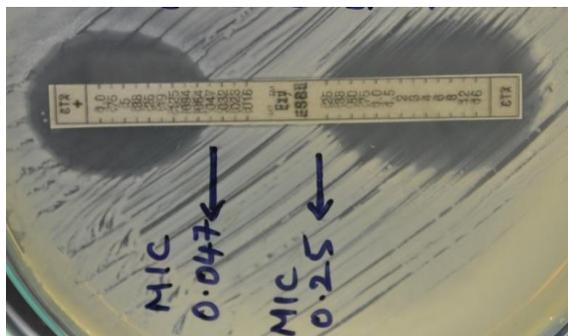
Microbiota isolated from pyometra pus samples includes *Escherichia coli* (7, 46.6%), *Staphylococcus* (11, 73.3%) and *Pseudomonas* (12, 80%) species. In a study by Bassessar *et al.*, (2013) from Madhya Pradesh, *E. coli* was isolated predominantly (50%) from canine pyometra cases followed by *Salmonella* (20%), *Staphylococcus* (20%) and *Pseudomonas* (10%) species. Coggan *et al.*, (2008) from Brazil reported 74.1% incidence of *E. coli* in canine pyometra cases. Microbiota isolated from otitis pus samples includes *Pseudomonas* (8, 53.3%), *Proteus vulgaris* (9, 60%) and *Staphylococcus* (15, 100%) species. *Staphylococcus* was by far

most common bacteria isolated from ear canal of dogs with otitis by many workers (Dixit *et al.*, 2011 and Petrov *et al.*, 2013). Dixit *et al.*, (2011) observed 37.1, 11.4 and 11.4% incidence of *Staphylococcus*, *Proteus* and *Pseudomonas* species respectively, from otitis cases of dogs.

E-test was performed for the detection of ESBL production in microbiota isolated in the present study (Fig. 1). Interpretation of results was done as per the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Table 1). Among the seven *E. coli* isolates, three (43%) isolates were found positive for simultaneous production of ESBL and AmpC (no zone is obtained for CTX and zone obtained in CTX+), two (28.5%) isolates were found ESBL negative (MIC of CTX < 0.5 and

the ratio of the MIC value of CTX / CTX+ is less than or equal to 8) and the remaining two (28.5) isolates gave non-conclusive results (no zone of inhibition on either side). Resistance in these isolates may be due to mechanisms other than ESBLs such as metallo beta-lactamases and porins (that are not inhibited by beta-lactamase inhibitors) which have to be further investigated before reporting (O'Keefe *et al.*, 2010). The co-existence of both ESBL and AmpC beta-lactamases could be due to dissemination of plasmid mediated AmpC beta-lactamases among Gram negative bacteria (O'Keefe *et al.*, 2010). The three *E. coli* isolates found positive for concurrent production of ESBL and AmpC enzymes in the present study were recovered from pus swabs of pyometra cases.

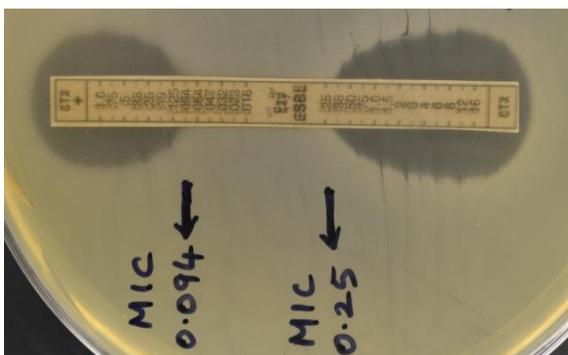
Fig.1 (A) & (B). E-test ESBL negative result with MIC ratio of (CTX): (CTX+) < 8; (C). E-test result showing production of both ESBL and AmpC enzymes (No zone is obtained for CTX and zone obtained in CTX+); (D). E-test non-conclusive result (No zone of inhibition on either side).



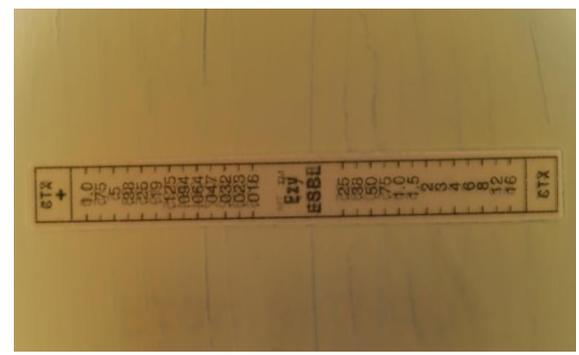
(A)



(C)



(B)



(D)

Table.1 Interpretation of ESBL E-test results

S. No.	Result	Interpretation
1.	CTX \geq 0.5 and the ratio of the MIC value of (CTX):(CTX+) is more than 8	ESBL positive
2.	Phantom zone just below the lowest concentration of CTX+ gradient or a deformation zone of the CTX inhibition ellipse at the tapering end	ESBL positive
3.	No zone is obtained for CTX and zone obtained in CTX+	Production of both ESBL and AmpC
4.	CTX $<$ 0.5 and the ratio of the MIC value of (CTX):(CTX+) is less than or equal to 8	ESBL negative
5.	No zone of inhibition on either side	Non-conclusive.

Among the 20 *Pseudomonas* isolates, 16 (80%) isolates were found ESBL negative (CTX $<$ 0.5 and CTX / CTX+ \leq 8) and the remaining four (20%) isolates gave non-conclusive results (no zone of inhibition on either side). All the 26 *Staphylococcus* and nine *Proteus* isolates recovered in the present study were found negative for ESBL production (CTX $<$ 0.5 and CTX / CTX+ \leq 8). O'Keefe *et al.*, (2010) from United States reported detection of ESBL production in 11 (18.3%) out of 60 *E. coli* isolates recovered from urinary tract infections in dogs using E-test. Tamang *et al.*, (2012) observed a positive confirmatory test for the production of ESBLs in 12 out of 18 cefotaxime resistant *E. coli* isolates recovered from stray dogs in Korea. Huber *et al.*, (2013) from Switzerland, reported 8.4% incidence of ESBL phenotype among *E. coli* isolated from dogs with urogenital infections. The sensitivity of E-test for the detection of ESBLs has been shown to vary from 94-100% (Drieux *et al.*, 2008 and Prabha *et al.*, 2016).

This study provides the first report of detection of concurrent production of both ESBL and AmpC enzymes in *E. coli* isolates (43%) recovered from canine pyometra cases in India using E-test. The presence of an ESBL-producing organism in a clinical

infection can result in treatment failure if extended spectrum cephalosporins and monobactams are used in the therapeutic regimens. It emphasize the clinical importance of accurate and early detection of resistant strains in companion animals in order to select the appropriate therapeutic options.

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