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Occurrence of ESBL producing *Yersinia enterocolitica* among companion animals in Wayanad district

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Abstract
Pet associated zoonotic diseases is one of the unexplored sector and *Yersinia* spp. especially antibiotic resistant strains can be a major public health. Hence, the present study was envisaged to evaluate the occurrence and characterization of ESBL producing *Y. enterocolitica* from dogs and cats of Wayanad district. A total of 100 faecal samples/swabs from dogs (50) and cats (50) were analyzed through conventional culture and molecular method using 16S rRNA PCR-RFLP. The occurrence of *Y. enterocolitica* was recorded in 12 samples. Species specific-PCR revealed that 12 per cent of samples were identified as *Y. enterocolitica* (ystA). Further, characterization of isolates for ESBL production through phenotypic disc diffusion method revealed that 41.66% of *Y. enterocolitica* isolates were ESBL producers and on genotypic PCR assay it was observed that 33.33% of isolates in dogs were found to harbour *bla* TEM gene. Likewise, 50% of Yersinia isolates (each) recovered from dogs were found to harbour *bla* SHV and *bla* CTX-M ESBL genes. Thus, the present study signifies the importance of virulent Yersinia strain harbouring ESBL resistance genes and its dissemination in companion animals which can have the potential to transfer to man.

Keywords: Dogs, cats, *Yersinia enterocolitica*, ESBL, 16S rRNA PCR-RFLP

1. Introduction
Companion animals such as dogs and cats play an important role in modern society. Apart from proven benefits of human-animal interactions, this may also enhance the risk of acquiring animal carried diseases (Kantere *et al*., 2014) [1]. The companion animals are potential carriers of zoonotic enteric bacterial pathogens and may lodge these pathogens internally in their gut as well as externally on their fur, paws, or head. The close association of humans with the household pets has imperiled the individuals' susceptibility to the pathogen (Belas *et al*., 2014) [2] *Y. enterocolitica* are Gram-negative, pleomorphic, non-spore forming rods or coccoid belong to the family *Enterobacteriaceae.* The organism is motile with peritrichous flagella and grows at optimum temperature between 22-30 °C on selective media (Fàbrega *et al*., 2012) [3]. *Y. enterocolitica* is the most commonly observed predominant species of *Yersinia*, often considered as a cause for infectious diarrhoea among animals and humans (Frazão *et al*., 2015) [4].
The molecular detection and identification of organisms of family *Enterobacteriaceae* can be performed using 16S rRNA PCR amplification followed by restriction enzyme (RE) digestion analysis (Vergis *et al*., 2013) [9]. A heat stable enterotoxin of *Y. enterocolitica* known as *yst*A, which is chromosomally encoded, is of diagnostic importance and could be used for detection of the organism in stool samples of humans and animals (Fàbrega *et al*. 2012) [3].
Resistance to third generation cephalosporins is documented among organisms of family *Enterobacteriaceae* and is due to the production of Extended-spectrum β-lactamase (ESBL) which confers resistance to broad-spectrum cephalosporins. The CTX-M, SHV and TEM variants of ESBL enzymes are most commonly reported among companion animals (Ewers *et al*., 2012). Moreover, colonization of multidrug resistant bacteria among animals is because of horizontal antimicrobial resistance gene transfer due to inappropriate usage of antibiotics during therapeutic and prophylactic dosage administration (Guardabassi *et al*., 2004) [6]
Studies to explore the occurrence of *Y. enterocolitica* and its potential in harbouring plasmid-borne resistance genes remains a major public health concern. Thus, the study was envisaged with the objective to explore the occurrence of bacterial pathogen *Y. enterocolitica* and characterization of recovered isolates for ESBL production

2. Materials and methods

2.1 Sample collection

A total of 100 faecal samples/swabs (50 each) from apparently healthy dogs and cats were collected from selected hospitals and veterinary dispensaries from three taluks of Wayanad district (Kerala) namely, Vythiri, Sultan Bathery and Mananthavady. The samples were collected in Cary-Blair transport media, aseptically packed in an insulated chilled condition and were processed on the same day in the laboratory for isolation, molecular detection and resistant gene characterization of *Y. enterocolitica*.

2.2 Isolation and Identification of *Y. enterocolitica* using culture method

Microbiological isolation of pathogen was performed by following defined protocol as described by Agarwal *et al*. (2003) [7] and (Fredriksson-Ahomaa, 2012). This includes enrichment of sample in a buffered peptone water (BPW) at 37 °C for 18 hours For selective isolation of *Y. enterocolitica*, the growth in BPW was further enriched in Irgasan-Ticarcillin-Chlorate (ITC) broth and a loopfull of this enriched culture was streaked onto Cefsulodin-Irgasan-Novobiocin (CIN) agar and incubated at 22-30° C for 24 hours where the organism was observed to be translucent colonies with dark pink center and bile precipitate The presumptive colonies were confirmed by performing Biochemical test that included IMViC test (Agarwal *et al*. 2003) [7].

2.2 PCR-RFLP based confirmation at genus level

Further confirmation of presumptive colonies from selective plate was done using molecular method by RE digestion analysis of 16S rRNA PCR product F:5'-TGCCTAATACATGCAAGTCG-3', R:5'-CGAATTAAA CCACATGCTC-3' oligonucleotide primer of 919bp amplicon size. The enzymes to differentiate the genus of enteric bacterial pathogen were selected using NEB cutter V2.0 (Vergis *et al*., 2013) [9]. The enzymes *Sal*I, *Eco*RI and *Hind*III were used with their respective buffers in the reaction mixture.

For DNA isolation, the overnight grown culture in BPW (1 ml) was centrifuged at 1000 × g for 10 min. The pellet was washed thrice in a PBS and centrifuged at 1000 × g for 10 min and then that pellet was suspended in PBS and subjected to vigorous heating in a boiling water bath at 100 °C for 10 min. This was immediately snap chilled on ice for 5 min, thawed and centrifuged again at 1000 × g for 5 min and supernatant (template) was stored at -20 ◦C.

Automated thermal cycler (Bio-Rad, USA) was used to carry out the PCR reactions. Based on the trials, the factors such as oligonucleotides, MgCl2, d NTP concentration, annealing temperature and PCR cycles were varied to arrive at an optimum PCR condition. The cycling conditions for 16S rRNA PCR included an initial denaturation at 94 ˚C for 5 minutes followed by 34 cycles each of 40 sec denaturation at 94 °C, annealing temperature at 59 °C and extension at 72 °C followed by a final extension of 5 minutes at 72 °C and final hold at 4 °C.

2.3 PCR confirmation of *Yersinia* spp.

For confirmation of *Y. enterocolitica*, *yst*A gene F:5'-AATGCTGTCTTCATTTGGAGC-3', R:5'-ATCCCAAT CACTACTGACTTC-3' was targeted. (Van Coppenraet *et al*., 2007) the PCR condition included initial denaturation at 94˚C for 5 minutes followed by 35 cycles of 40 sec denaturation at 94 °C, annealing temperature at 58 °C for 50 sec and extension at 72 °C for 50 sec followed by a final extension of 5 minutes at 72 °C and final hold at 4 °C.

2.4 Phenotypic assay for ESBL production

The antibiotic susceptibility testing was carried from the *Y. enterocolitica* isolates as per the guidelines provided by Clinical Laboratory Standards Institute (2018) [11]. The bacterial isolates were subjected to standard disc diffusion method (Nagdeo *et al*., 2012) [12]. Antibiotics discs used in the study were procured from HiMedia Laboratories Ltd., Mumbai. *Escherichia coli* ATCC®25922 was the quality control strain used in the study. Commercial antibiotic discs, Aztreonam (30 µg), Ceftriaxone (30 µg), Cefotaxime (CTX 30 µg), Ceftazidime (CAZ 30 µg), Ceftazidime/ Clavulanic acid (CAC 30/10 µg), Cefoxitin (30 µg) and Imipenem (10 µg) (HiMedia) were used in the study.

Pure culture of test isolate equivalent to 0.5 McFarland approximately 1.5 $\times 10^8$ CFU/ml was evenly spread on Mueller-Hinton (MH) agar plate using a sterile cotton swab. Antibiotic discs were placed on the inoculated agar surface at about two to three cm apart with gentle pressure and incubated for overnight incubation at 37 °C. The zone of inhibition diameter was measured for each antibiotic the obtained data was compared with interpretative chart furnished by the manufacturer to grade the test isolates as sensitive, intermediate and resistant for respective antibiotics.

2.5 Genotypic assay for ESBL production

Singleplex PCR was standardized for identification and characterization of recovered positive isolates for presence of ESBL producing genes viz., *bla*CTX-M gene F:5'-CGCTTTGCGATGTGCAG-3', R:5'-ACCGCGATATCGT TGGT-3' oligonucleotide of amplicon size 550bp (Ahmed, 2003). *bla*SHV gene F:5'-GATGAACGCTTTCCCCATG ATG-3', R:5'-CGCTGTTATCGCTCATGGTAA-3' oligo - nucleotide of amplicon size 214bp and *bla*TEM F:5'-ATG AGTATTCAACATTTCCG-3', R:5'-GTCACAGTTACCA ATGCTTA-3' of amplicon size 847bp (Yazdi *et al*., 2012) [14]. The PCR condition involved the steps as; initial denaturation of DNA at 94 ˚C for 5 minutes followed by 35 cycles each of 1 min denaturation at 94 °C, annealing temperature at 59 °C and extension at 72 °C followed by a final extension of 5 minutes at 72 °C and final hold at 4 °C.

2.6 Statistical analysis

The data obtained were subjected to statistical analysis following procedures described by Snedecor and Cochran (1994) [15] using the SPSS software version 24.0 chi-square test for multiple proportions was performed.

3. Results

The present study was envisaged to evaluate the prevalence of *Y. enterocolitica* a major enteric bacterial pathogen in dogs and cats of Wayanad district, and to characterize the ESBL resistance of this pathogen. Samples comprised of total of 100 faecal samples/swabs (50 each) were collected from dogs and cats from Vythiri, Sultan Bathery and Mananthavady Taluks of Wayanad district.

Out of 100 faecal samples/swabs from dogs and cats analysed, the microbiological isolation protocol could detect translucent colonies with dark pink center and bile precipitate on CIN agar which were presumptively identified as *Y. enterocolitica*

(fig. 1). The colonies from selective plates were further subjected to biochemical identification using IMViC test reaction revealed + - - - reading for the samples. For, genus level identification, presumptive colonies from selective plates were amplified using 16S rRNA oligonucleotide primer followed by RE digestion using restriction endonucleases enzymes *Eco*RI, *Sal*I and *Hind*III. The results revealed the restriction digestion pattern of product size 630/289 for *Eco*RI, 777/89/53 for *Sal*I and no cut sites for *Hind*III (fig. 2) that was observed in twelve samples. Of this 12 samples, six were from dogs and six were from cats which were in accordance with biochemical test.

Further species-specific PCR for *Yersinia* spp. by targeting *yst*A gene revealed that all 12 isolates recovered from dogs and cats carried the gene *yst*A (fig. 3). Thus, delineating the occurrence of *Y. enterocolitica* as 12% each in dogs and cats. The taluk-wise occurrence revealed that 10% of the total samples were from Vythiri and the 10% of the total samples from Mananthavady taluks. The highest occurrence 16.66% of *Y. enterocolitica* was noticed in samples from Suthan Bathery taluk. However, the chi-square values for multiple proportions was found to be non-significant, no significant difference was observed in the occurrence of *Y. enterocolitica* between species and also between each taluks of Wayanad district (Table: 1).

Antibiotic susceptibility test, phenotypic assay for identification of ESBL producers, from the above 12 isolates recovered from dogs and cats analysed by standard disc diffusion method revealed that 41.66% of isolates were found to be ESBL producers and were exhibiting phenotypic resistant pattern to cefotaxime and ceftazidime. However, most of the isolates were sensitive to imipenem, aztreonam, cefotaxime/ clavulanic acid and ceftriaxone in descending order (fig. 4). Genotypic characterization of positive *Y. enterocolitica* isolates targeting *bla*CTX-M, *bla*SHV and *bla* TEM revealed that 33.33% (2 isolates) recovered from dogs were found to harbour *bla* CTX-M gene (fig.5) and 50% (3 isolates from dogs each) were harbouring *bla* SHV (fig.6) and *bla* TEM (fig.7) genes. However, ESBL conferring genes were not observed in isolates recovered from cats.

4. Tables and Figures

Table 1: Taluk wise occurrence of *Y. enterocolitica*

Sl No.	Taluk	No. of samples	*Y. enterocolitica*	
			Positive	Percentage
1	Vythiri	40	4	10
2	Mananthavady	30	3	10
3	Sulthan Bathery	30	5	16.66
Total		100	12	12
Chi-Square value		0.884^{ns}		
p-value		0.643^{ns}		

ns-nonsignificant

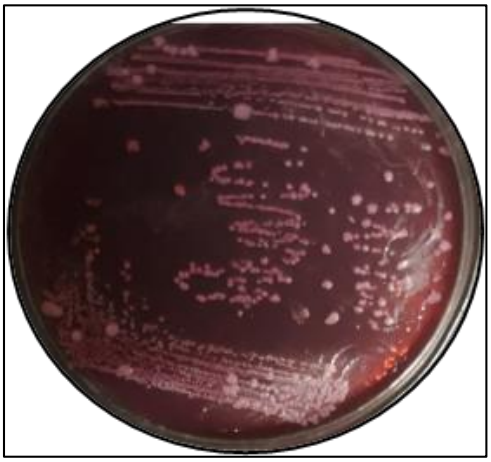
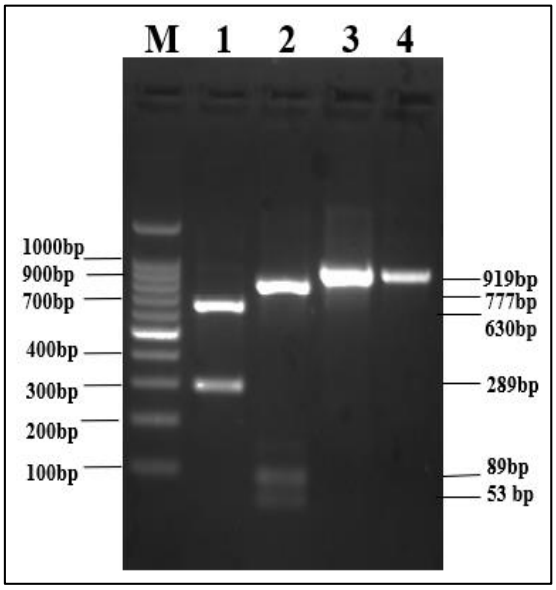


Fig 1: CIN agar *Y. enterocolitica* translucent colonies with dark pink center and bile precipitate



***Yersinia* spp.**

Fig 2: PCR Standardization of 16S rRNA PCR-RFLP, Lane M-Marker, Lane1-*Eco*RI, Lane2-*Sal*I, Lane3- *Hind*III, Lane4-Undigested 16SrRNA product of *Yersinia* spp.

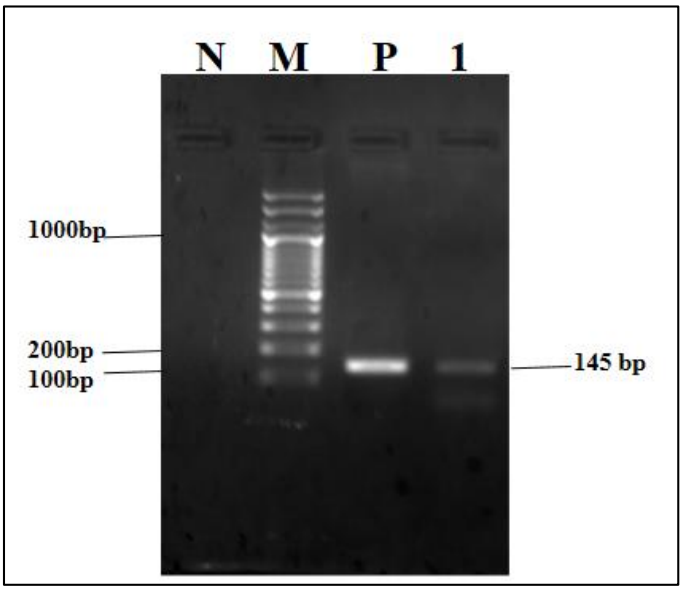


Fig 3: PCR Standardization of *yst*A gene, Lane M-Marker, Lane N-Negative control, Lane P-Positive control, Lane1- samples of amplicon size 145bp

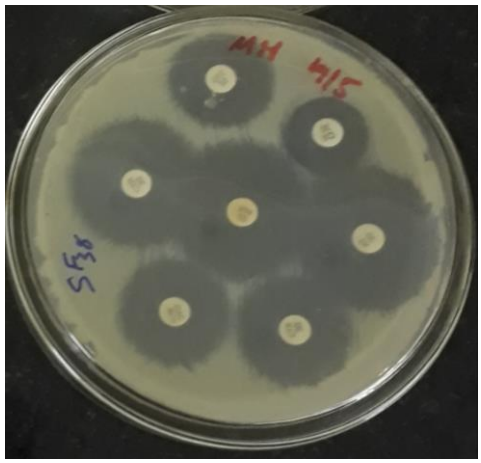


Fig 4: Antibiotic Susceptibility profile of isolates under standard disc diffusion

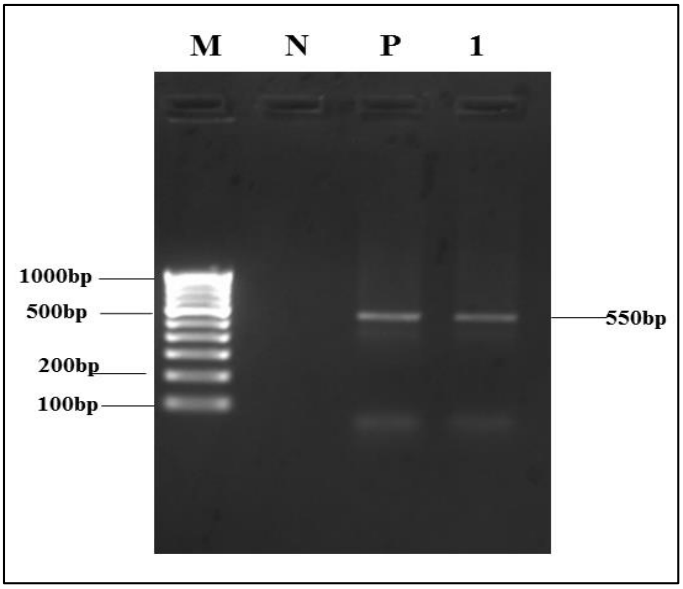


Fig 5: PCR Standardization of *bla*CTX-M gene, Lane M-Marker, Lane N-Negative control, Lane P-Positive control, Lane1- samples of amplicon size 550bp

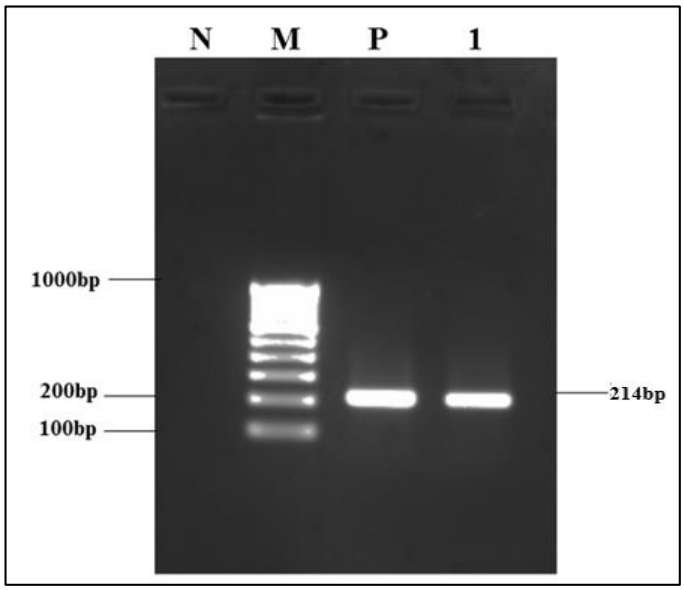


Fig 6: PCR Standardization of *bla* SHV gene, Lane M-Marker, Lane N-Negative control, Lane P-Positive control, Lane1- samples of amplicon size 214bp

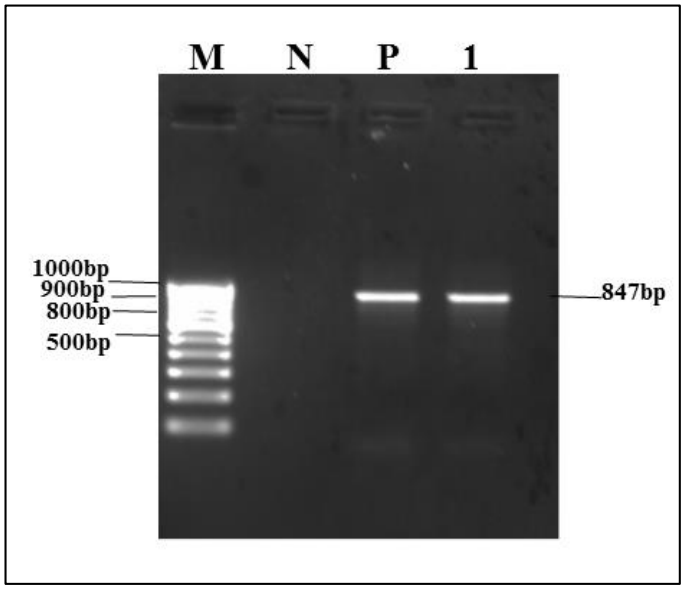


Fig 7: PCR Standardization of *bla* TEM gene, Lane M-Marker, Lane N-Negative control, Lane P-Positive control, Lane1- samples of amplicon size 847bp

5. Discussion

The present study was carried out with the objective to explore the occurrence of *Y. enterocolitica* an enteric bacterial pathogen, and to know their ability for production of ESBL. In order to represent companion animals that have close association with man, the study deliberately chose dogs and cats as the subject. For the microbiological isolation and detection of *Y. enterocolitica*, selective enrichment in ITC broth followed by plating onto CIN agar to get red bull's-eye colonies was adapted which is considered as the best suitable method by Fredriksson-Ahomaa, 2012. Further confirmation of presumptive colonies by biochemical identification identified six isolates each from dogs and cats as *Yersinia* spp. The results of biochemical tests were also in accordance with 16S rRNA PCR-RFLP analysis. The similiar reports of molecular detection of organisms of family *Enterobacteriaceae*, was performed from researchers viz., Ab-Ed-Haleem *et al*., 2003; Bouzari *et al*., 2011 and Vergis *et*

al.,2013 [16, 17, 9].

The molecular characterization for the presence of *yst*A gene of *Y. enterocolitica* among dogs and cats could also 12% (6 samples each) from dogs and cats were found harbouring the gene. Similarly, the earlier studies conducted by Stamm *et al*. (2013) [18] in pet animals could report *Y. enterocolitica* in 4.6 per cent of samples from dogs and 0.3 per cent of samples from cats which showed a rate that is much lower when compared to the present study.

The phenotypic characterization of total of 12 *Y. enterocolitica* isolates for ESBL production revealed that 41.66% of isolates were resistant to cefotaxime and ceftazidime and were identified as ESBL producers. Similarly, a study conducted by Frazão *et al*. (2017) [19] reported that 41.17 per cent of *Yersinia* isolates recovered from companion animals as ESBL producer that were identified through phenotypic double disc synergy test. The results obtained in the present study was comparable with earlier authors.

Genotypic characterization for ESBL producers of *Y. enterocolitica* isolates recovered from companion animals revealed that 28.57 per cent of isolates were ESBL producers. The isolates recovered from dogs were found to harbour ESBL encoding genes. However, no isolate from cats harboured this gene. A study conducted by Bent and Young (2010) [20] also reported the occurrence of β-lactamase enzymes among *Y. enterocolitica* serovars.

The present study could able to detect *Y. enterocolitica* an enteric bacterial pathogen of public health significance among companion animals. ESBLs enzymes *bla* CTX-M, SHV and TEM conferring resistance to third generation cephalosporins were also identified among isolates through standard microbiological and molecular methods.

6. Conclusions

The study thus signifies the potential of these animals to act as sources of infections for transmission of resistant pathogens to humans and other animals, at the same time pointing to the need for adopting strict public health measures to prevent the spillover from population to population.

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