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Molecular Detection of Extended Spectrum Beta Lactamase (ESBL) Producing Multidrug Resistant *E. coli* from Rabbit

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ABSTRACT

In the present study a lung sample was collected from autopsied rabbit, which was suspected to have died due to *E. coli* infection and cultured on MacConkey agar. Itrevealed typical cultural and biochemical characteristics of *E. coli*. Molecular confirmation of *E. coli* was carried out using 16s RNA (ECO1) universal eubacterial primers with positive 585 bp amplicon**.** A total 26 Antibiotic disc used for antibiotic sensitivity test. Out of 26 Antibiotic disc, only two antibiotics (Imipenem and Cefoxitin) sensitive against isolated *E. coli*. Phenotypic characterization of ESBL by combine disk diffusion method. The difference between zone of inhibition is 20 mm around the combined disk containing clavulanic acid then the corresponding disk with Ceftazidime and cefotaxime is indicate positive for ESBL producing *E. coli*. Upon genotypic conformation of ESBL, the isolate was found positive for CTX-M1, CTX-M2, and TEM and negative for CTX-M9, SHV and OXA genes.

HIGHLIGHTS

- Multi drug resistant ESBL producing *E. coli* was isolated from rabbit.
- Harboring of CTXM-1, CTXM-2 and TEM genes and absence of CTX-M9, SHV and OXA genes were confirmed by PCR.

Keywords: *Escherichia coli*, ESBL, Multidrug resistant, PCR, Rabbit

Emergence of multidrug resistance (MDR) *E. coli* is a major concern for human and animals due to their rapid spread. MDR is linked with high disease burden and economic consequences on people and nations. Continuous surveillance of antimicrobial resistance in bacteria that cause infections in humans and animals is essential to know the antimicrobial susceptibility dynamics of these organisms over the time. Extended spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem). Such enzymes have been most commonly detected in *E. coli* and *Klebseilla pneumoniae*. ESBLs are inhibited by inhibitors such as clavulanic acid, tazobactam and sulbactam. There are more than 200 different types of ESBL enzymes of which TEM, SHV and CTX-M types are more frequent (Rahman *et al.,* 2017). Its ubiquitous presence and wide host range justifies its designation as a sentinel organism for antimicrobial resistance development in different types of animals (Ashbolt *et al.,* 2013). Sufficient concern has though been given towards the emergence and spread of extended spectrum β-lactamase (ESBL) producing *E. coli*

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associated with cattle and other farm animals(Bush and Jacoby, 2010; Pfeifer *et al.*, 2010), but there is very few reports about ESBL producing *E. coli* in laboratory animals like rabbit, rat, mice *etc*. A steady increase of these strains have been reported worldwide and in this connection, the present communication givesa report of ESBL producing *E.coli* in rabbit. Best of author's knowledge this is the first report of presence of ESBL in rabbit from India.

MATERIALS AND METHODS

In the present study, a lung sample was aseptically collected from necropsied rabbit. Necropsy findings suggested *E. coli* infection as cause of death. The sample was streaked on MacConkey agar and EMB agar medium and incubated aerobically at 37°C for overnight. The colony morphology was observed and the features of microorganisms were studies using Gram's staining method. Further isolate was confirmed by biochemical tests like Indole, Methyl red, Voges-Proskaur, Citrate utilization and Oxidase as per process recommended by Edwards and Ewing (1972). A drug sensitivity pattern was studied by disk diffusion method following the method of Bauer *et al.* (1966). In total 26 different antibiotics commonly used for animal treatments were used in the study (Table 1).

Phenotypic characterization of ESBL was done by combine disk diffusion method (CLSI VET08Ed4E). The difference between zone of inhibition is ≥5 mm around the combined disk containing clavulanic acid then the corresponding disk with Ceftazidime and cefotaxime is indicate positive for ESBL production.

For molecular characterization genomic DNA was extracted from the freshly grown culture by heat method and the species of the isolate was confirmed by PCR using species-specific 16S rRNA primer ECO-1 (Fratamico *et al*., 2000). Genotypic evaluation of ESBL production was carried out by detecting CTX-M-1, CTX-M-2, CTX-M-9, TEM, OXA and SHV using gene specific primer (Dallenn *et al.,* 2010; Koovapra *et al*., 2016). PCR was standardized in a total reaction volume of 25 µl for each gene, containing 12.5 µl of 2X PCR master mixture, 10 pmol of forward and reverse primers each (Table 2), 2 µl DNA as template and nuclease free water up to 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany) as per table 3. The amplified product was electrophoresed in 1.5% agarose gel stained with ethidium bromide (0.5 µg /ml) and image was documented by gel documentation system (Mini BiSBio Imaging System).

RESULTS AND DISCUSSION

The organisms were successfully isolated from the rabbit's lung tissue showing characteristic lactose fermenting pink coloured colonies and greenish metallic sheen on MacConkey agar and eosin methylene blue (EMB) agar, respectively. On gram's staining organisms looks like gram negative bacilli, whereas in biochemical tests it shows negative for oxidase test and + + - - pattern for IMViC test. In antibiotic sensitivity test only two antibiotics *viz*. Imipenem and Cefoxitin were found sensitive against the isolate. In phenotypic characterization of ESBL by combine disk diffusion method difference between zone

Table 1: List of Antibiotic disc used for Antibiotic Sensitivity Test and its result against Isolated *E.coli*

Sl. No.	Name of Antibiotic	Result	Sl. No.	Name of Antibiotic	Result
1	Enrofloxacin	Resistant	14	Ciprofloxacin	Resistant
2	Ceftriaxone	Resistant	15	Gentamicin	Resistant
3	Imipenem	Sensitive	16	Colistin	Resistant
4	Cefoxitin	Sensitive	17	Moxifloxacin	Resistant
5	Amikacin	Resistant	18	Cefdinir	Resistant
6	Tetracycline	Resistant	19	Ampicillin	Resistant
7	Amoxyclav	Resistant	20	Cefepime	Resistant
8	Chloramphenicol	Resistant	21	Doxycycline	Resistant
9	Cefpodoxime	Resistant	22	Cefotaxime	Resistant
10	Nalidixic acid	Resistant	23	Ceftazidime	Resistant
11	Levofloxacin	Resistant	24	Oxytetracycline	Resistant
12	Methicillin	Resistant	25	Ceftizoxime	Resistant
13	Aztreonam	Resistant	26	Co-trimoxazole	Resistant

Table 2: Details of primers for amplification of ECO1, CTX-M1, CTX-M2, CTX- M9, TEM and SHV genes of *E. coli*

Gene designated	Primer sequence (5'- 3')		Size of amplified products (bp)
ECO1 16sRNA	F	GACCTCGGTTTAGTTCACAGA	585
	R	CACACGCTGACGCTGACCA	
CTX-M-1	F	TTAGGAARTGTGCCGCTGYA	688
	R	CGATATCGTTGGTGGTRCCAT	
CTX-M-2	F	CGTTAACGGCACGATGAC	404
	R	CGATATCGTTGGTGGTRCCAT	
CTX-M-9	F	TCAAGCCTGCCGATCTGGT	561
	R	TGATTCTCGCCGCTGAAG	
TEM	F	CATTTCCGTGTCGCCCTTATTC	800
	R	CGTTCATCCATAGTTGCCTGAC	
SHV	F	AGCCGCTTGAGCAAATTAAAC	713
	R	ATCCCGCAGATAAATCACCAC	
OXA	F	GGCACCAGATTCAACTTTCAAG	564
	R	GACCCCAAGTTTCCTGTAAGTG	

Table 3: Steps and conditions of thermal cycling for various PCR

Primers	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
ECO-1	94ºC 5 min.	94ºC 30 sec.	53ºC 1 min.	72ºC 1 min.	72ºC 8 min.
CTX-M-1, CTX-M-2, CTX-M-9, TEM, SHV and OXA gene	94ºC 10 min.	94ºC 40 sec.	60ºC 40 sec.	72ºC 1 min.	72ºC 7 min.
		For 30 cycles			

of inhibition was 20 mm which is indicative of ESBL producing *E. coli* (Fig. 1).

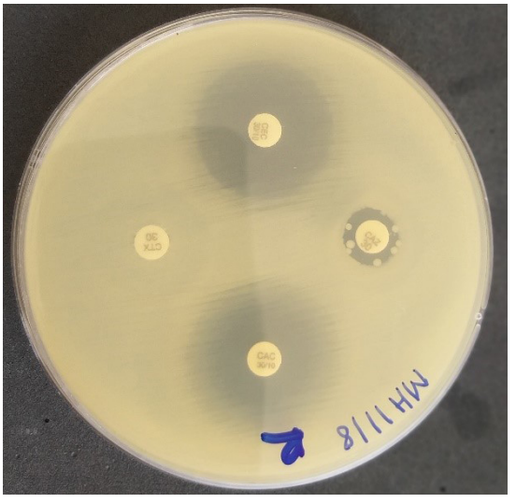

Fig. 1: Phenotypic demonstration of ESBL producing *Escherichia coli* by combine disk diffusion method showing inhibition of ≥5 mm around the combined disk containing antibiotic and clavulanic acid than the corresponding antibiotic disk

Molecular confirmation of *E. coli* isolates was carried out using 16s RNA universal eubacterial primers (ECO-1) which shows a amplification of target band of 585 bp size which is indicative of *E.coli*. The isolate was screened for presence of CTX-M1, CTX-M2, CTX-M9, TEM, OXA and SHV genes using PCR and found positive for CTXM-1 (688 bp), CTXM-2 (404 bp) and TEM (800 bp) genes (Fig. 2, A-D); whereas it found negative for CTX-M9, SHV and OXA genes. Brinas *et al.* (2002) reported *E. coli* is the commonest bacteria which causes intestinal and extra intestinal infection in different animals and humans with ESBL. Zaho *et al.* (2018) and Silva *et al.* (2010) isolate multidrug resistance *E. coli* from cases of collibacillosis from domestic rabbits and wild rabbits, respectively. But in the present study it was recovered from extra intestinal tissue. In present study, ESBL enzymes and their genes have been demonstrated with both phenotypic and

molecular methods, respectively. Though these were not reported in few earlier studies. In china Zaho *et al.* (2018) reported 50.90 % MDR-ESBL producing *E. coli*, It also detect 98.20% and 94.50% β-lactamase genes, *bla*TEM and *bla*CTX-M respectively form 55 Isolated *E.coli* from rabbit, but they had isolated it from fecal sample. Pandor (2019) reported out of 25 ESBL *E. coli* isolates, 19 (35.85%), 8 (15.09%) and 7 (13.21%) isolates positive for TEM, SHV and CTX-M gene, respectively. Whereas 6 (15.09%) isolates harbored two genes i.e., CTX-M and TEM; TEM and SHV and 3 (5.66%) isolates harbored all the three genes CTX-M, TEM and SHV. This indicate spread of such deleterious genes in laboratory animal population. Similar to Brinas *et al*. (2002) and Zaho *et al*. (2018). CTX-M and TEM genes were detected in this work which are said to be commonest among ESBL genes whereas SHV and OXA genes were less prevalent among population, as previously recorded by Brinas *et al*. (2002).

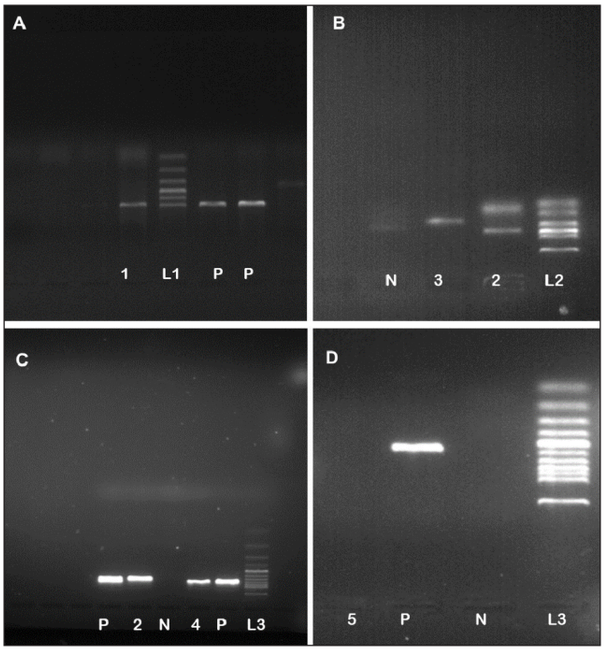

Fig. 2: PCR based confirmation of *E. coli* (A) and presence or absence of antibiotic resistant genes (B-D)

Plate A – Gene Pilot 100 bp ladder (L1) and positive samples for ECO1gene; **Plate B** – Gene Pilot 100-2000 bp ladder (L2) and positive samples for CTX1 gene (2) and CTX2 gene (3); **Plate C** – Gene Pilot 100 bp ladder (L3) and positive samples for CTX1 gene (2) and TEM gene (4); **Plate D** – Gene Pilot 100 bp ladder (L3) and negative samples for OXA gene (5)

CONCLUSION

It was concluded that the rabbit was died due to ESBL producing multidrug resistant *E. coli*. The occurrence of MDR strains is a potential threat not only to animal health but also to workers associated with maintenance of lab animals' colonies.

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