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Occurrence and Characterization of Extended Spectrum β- lactamase Producing *E. coli* and *Salmonella* spp. from Raw Milk Samples in Wayanad District, Kerala, India

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A B S T R A C T

Escherichia coli and *Salmonella* are considerably responsible for causing gastrointestinal disorders. Emergence of Extended spectrum beta lactamase (ESBL) production among the above organisms is a great matter of concern. Hence, the present study is envisaged to know the distribution status of ESBL producing *E. coli* and *Salmonella* spp. from raw milk samples. A total of 50 raw milk samples was collected and were analyzed for the *E. coli* and *Salmonella* through conventional culture method and confirmation by PCR using *uid*A gene (*E. coli*) and *inv*A gene (*Salmonella).* The occurrence of *E. coli* and *Salmonella* spp in raw milk was found to be 60 per cent and 18 per cent, respectively Further, characterization of isolates for phenotypic ESBL production revealed that 21 *E. coli* (70%) and 7 *Salmonella* (77.77 %) as phenotypic ESBL producers and were resistant to third generation cephalosporins. *bla*TEM, *bla*CTX-M *and bla*SHV gene was present in 56.66 per cent, 40 per cent and 26.66 per cent of the *E. coli* isolates and the occurrence of *bla*SHV, *bla*CTX-M and *bla*TEM gene in *Salmonella* isolates was 55. 5 percent, 44.4 per cent and 11.1 per cent, respectively. Thus the present study envisaged the need of hygienic milk production and proper antibiotic usage to prevent further spillover of resistant pathogenic organisms among public and environment.

Introduction

Milk is a highly nutritious food. However, it acts as a good medium for the growth of different microorganisms which cause spoilage of milk leading to severe infections in humans (Murinda *et al.,* 2004). Generally contamination of milk occurs through colonization of teat canal by various pathogenic microorganisms like *E. coli* and *Staphylococcus aureus* which are commonly present in environment or might be due to

external contamination from milkers hand, external environment and water used during milking (Thaker *et al.,* 2012). Contamination of raw milk with *Salmonella* organism is attributed to faecal contamination by infected animals, contaminated utensils or through contaminated water. There are some consumers who prefer raw milk and their products over pasteurized milk for consumption which can be a major public health concern if the milk gets contaminated (Chye *et al.,* 2004).

Indiscriminate use of antibiotics without antibiotic sensitivity test and misuse of antibiotics has led to the development of resistance to commonly used antibiotics among micro organisms which are shedding out through milk (Owens *et al.,* 1997).

Salmonella and pathogenic strain of *E. coli* are the leading cause of food borne zoonosis annually worldwide which spreads even through contaminated milk have acquired antibiotic resistance to commonly used antibiotics including third generation cephalosporins (Levy, 1997). From past 2 decades, Extended spectrum beta lactamases resistance has spread among pathogenic microorganisms all over the world which are having ability to hydrolyze newer antibiotics even third generation cephalosporins (ceftriaxone, cefotaxime, ceftazidime) and monobactams (aztreonam). Thus the study was envisaged to know the occurrence of ESBL producing *E. coli* and *Salmonella* organisms and their resistance pattern to various antibiotics from raw milk samples in Wayanad district.

Materials and Methods

Sample collection

A total of 50 raw milk samples were collected from milk societies and dairy farmers of Vythiri, Sulthan bathery and Mananthavady taluks of Wayanad district, Kerala. Samples were collected in sterile test tubes with screw capped and transported to laboratory in refrigerated condition.

E. coli and *Salmonella* isolation by culture method

Isolation of *E. coli* and *Salmonella* was carried out as per the standard protocol described by Agarwal *et al.,* (2003) and Barrow and Feltham (2003). Pre enrichment of the samples was done in Buffered peptone water, followed by enrichment at the rate of 1:10 dilution in nutrient broth for *E. coli* and selenite broth for *Salmonella*. This was followed by selective plating onto EMB agar (*E. coli*) and on HE agar (*Salmonella*). On EMB agar black/purple colonies with green metallic sheen were presumptively identified to be *E. coli* and black colour colonies with narrow green margin were presumptively identified as *Salmonella.* All the above mentioned colonies were subjected to biochemical tests for confirmation as per the procedure described by Barrow and Feltham (2003) and Agarwal *et al.,* (2003) by performing primary, secondary (IMViC test) and other tertiary biochemical tests (Fig. 1 and 2).

Molecular confirmation of *E. coli* and *Salmonella*

E. coli and *Salmonella* isolates obtained from culture method were confirmed by PCR targeting *uid*A gene and *inv*A gene, respectively. The primer details and cyclic condition of both genes are provided in Table 1 and 2.

Phenotypic and genotypic characterization of isolates for ESBL production

The antibiotic susceptibility testing of confirmed isolates of *E. coli* and *Salmonella*

was carried out as per the guidelines provided by Clinical Laboratory Standards Institute (2018). *Escherichia coli* ATCC 25922 was used as the quality control strain. Commercial antibiotic discs, Aztreonam (30 µg), Ceftriaxone (30 µg), Cefotaxime (CTX 30µg), Ceftazidime (CAZ 30µg), Ceftazidime/ Clavulanic acid (CAC 30/10µg), Cefoxitin (30µg) and Imipenem(10µg) (HiMedia Laboratories Ltd, Mumbai) were used in the study. Isolates were screened for ESBL production based on standard disc diffusion method as per Nagdeo *et al.,* (2012).

Briefly, sterile cotton swab was used to inoculate pure culture of test isolate (equivalent to 0.5 Mcfarland approximately 1.5×10^8 CFU/ml) evenly on Muller-Hinton (MH) agar. Antibiotic discs were placed on inoculated MH agar and incubated for overnight incubation at 37 °C. The zone of inhibition diameter was measured for each antibiotic and was compared with interpretative chart furnished by the manufacturer to grade the test isolates as sensitive, intermediate and resistant for respective antibiotics.

Genotypic characterization of the isolates by PCR targeting ESBL producing genes

PCR was standardized for characterization of the positive *E. coli* and *Salmonella* isolates for presence of ESBL producing genes. The details of the ESBL genes *bla*CTX-M, *bla*SHV and *bla*TEM are mentioned in Table 1.

PCR condition for ESBL genes involved the following steps, initial denaturation at 94˚C for 5 min followed by 35 cycles each of denaturation at 94 ˚C for 1 min, annealing at 56 ˚C for 1 min, extension at 72 ˚C for 1 min and final extension at 72 ˚C for 5 min. Automated thermal cycler (Bio-Rad, USA) was used to carry out the PCR reactions.

Results and Discussion

Occurrence of *E. coli* and *Salmonella* isolates from raw milk samples

In the present study occurrence of *E. coli* organism from raw milk samples collected from various taluks of Wayanad district was found to be 60 per cent (30 numbers) which is in accordance with the results obtained by Ali and Abdelgadir (2011) where the prevalence rate was 63 per cent. In contrast, a lower prevalence rate was recorded in the studies conducted by Thaker *et al.,* (2012) (38 per cent) and by Kumar and Prasad (2010) (6.66 per cent). Hence, contamination of milk with *E. coli* is comparatively higher in Wayanad district which emphasize the need to adopt hygienic practices at farms. The higher occurrence rate of *E. coli* further emphasizes the need to adopt strict hygienic measures at milk production and collection units including its transportation. There is a need to educate farmers on environmental contamination at cattle shed, hygienic milkers and milking practice, health animal/ udder and clean milk vessels. The prevalence rate of *Salmonella* among the collected raw milk samples was 18 per cent which is in accordance with the findings of Tadesse and Dabassa (2012) where the prevalence rate was 20 per cent. In contrary lower prevalence was observed by Karns *et al.,* (2005) (2.6 per cent) and Kaushik *et al.,* (2014) (5.6 per cent). The finding of a higher occurrence rate in our study might be because of external faecal contamination or by the usage of contaminated water at the time of milking.

Phenotypic characterization of *E. coli* and *Salmonella* isolates for ESBL production by disc diffusion method

A total of 30 *E. coli* and 9 *Salmonella* isolates were isolated from raw milk samples analyzed. Among them 21 *E. coli* (70%) and 7

Salmonella (77.77 %) isolates were found positive for phenotypic ESBL production where they exhibited resistance to cefotaxime and ceftazadime. Antibiotic susceptibility testing revealed that *E. coli* isolates showed more resistance to third generation cephalosporins like cefotaxime (60.00%) and ceftazadime (46.66%). They were more susceptible to antibiotics imipenum (76.66%), aztreonam (60.00%), ceftazadime/clavulanic acid (56. 66%) and ceftriaxone (46.66%) in descending order. Olowe *et al.,* (2015) in his research identified 63.2 per cent of the *E. coli* isolates as phenotypic ESBL producers which is similar to the results of present study. However, lesser prevalence rate was observed by Sharma *et al.,* (2013) in Jaipur (52.49 per cent). *Salmonella* isolates were more susceptible to imipenum (77.77%), ceftazadime/clavulanic acid (66.66%), ceftriaxone (55.55%) and aztreonam (44.44%) in descending order. They showed more resistance to cefotaxime (66.66%) and ceftazadime (33.33%). Higher isolation rate of ESBL producing *Salmonella* isolates were observed in the present study compared to Yang *et al.,* (2012) where 21.7 per cent of the *Salmonella* isolates were phenotypic ESBL producers.

Genotypic characterization of *E. coli* and *Salmonella* isolates for ESBL production

All the phenotypic ESBL producers were examined for the presence of various ESBL genes including *bla*CTX-M, *bla*SHV and *bla*TEM gene. *bla*TEM was present in 17 (56.66%) *E. coli* isolates, *bla*CTX-M was present in 12 (40.00 %) isolates and *bla*SHV was less prevalent and detected in 8 (26.66 %) isolates. Three *E. coli* isolates were found to possess all the three *bla* genes. *bla*CTX-M and *bla*TEM together seen in 8 *E. coli* isolates and it is the common combination seen. Hence, the present study showed *bla*TEM as the most prevalent gene among *E. coli* isolates in Wayanad district which is similar to the results of Bajpai *et al.,* (2017) where in, *bla*TEM was the most prominent gene with the prevalence rate of 48.7 per cent followed by *bla*CTX-M (7.6 per cent) and *bla*SHV gene (5.1 per cent).

Among nine *Salmonella* isolates recovered from raw milk samples, five (55.5 per cent) were harboring *bla*SHV gene, four (44.4 per cent) were harboring *bla*CTX-M gene and one (11.1 per cent) isolate harbored *bla*TEM gene (Fig. 3–7).

Table.1 Details of the primer used

Target organism	Gene Primer sequence	Amplicon size	Reference
E.coli	***uidA*** F:5'- TGGTAATTACCGACGAAAACGGC-3' R: 5'- ACGCGTGGTTACAGTCTTGCG-3'	162bp	(Bej, 1991)
Salmonella	***inv*A** F:5'-GTGAAATTATCGCCACGTTCGGGCAA-3' R: 5'-TCATCGCACCGTCAAAGGAAC -3'	284bp	(Galan *et al*., 1992)
ESBL producing *Enterobacteriaceae*	***bla*CTX-M** F:5'-CGCTTTGCGATGTGCAG-3' R: 5'-ACCGCGATATCGTTGGT-3'	550bp	(Ahmed *et al.,* 2004)
	***bla*SHV** F:5'GATGAACGCTTTCCCATGATG-3' R: 5'-CGCTGTTATCGCTCATGGTAA-3'	214bp	(Yazdi *et al*., 2012)
	***bla*TEM** F:5'ATGAGTATTCAACATTTCCG-3' R: 5'-GTCACAGTTACCAATGCTTA-3'	847bp	(Yazdi *et al*., 2012)

Table.2 PCR cyclic condition for *E. coli* and *Salmonella*

PCR conditions	*uid A* gene for *E. coli*		*inv A* gene for *Salmonella*	
Process	**Temp(◦C)**	**Time**	**Temp(◦C)**	**Time**
Initial denaturation	94	5 min	94	5 min
Denaturation	94	40sec	94	30 sec
Annealing	55	60 sec	55	40 sec
Extension	72	50 sec	72	50 sec
Repeated cycle of denaturation to extension 35 cycles				
Final extension	72	5 min	72	5 min

Fig.1 *Salmonella* isolates on HE agar (Black colonies with green margin)

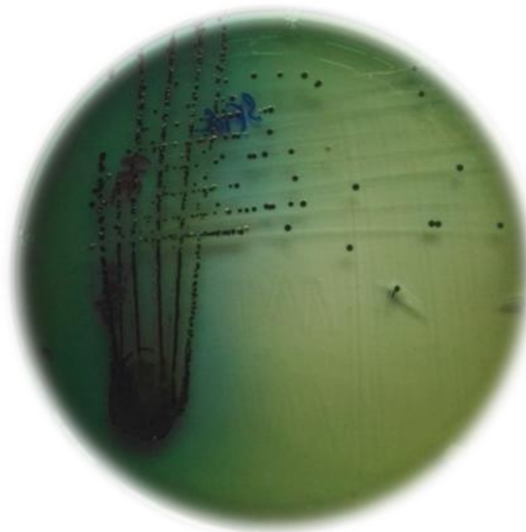


Fig.2 *E. coli* isolates on EMB agar (Purple colonies with green metallic sheen)

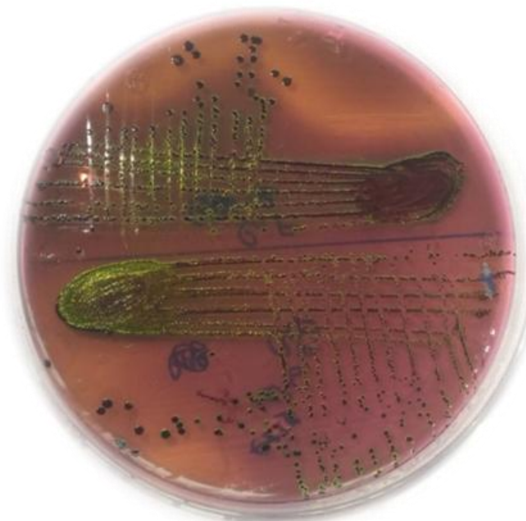
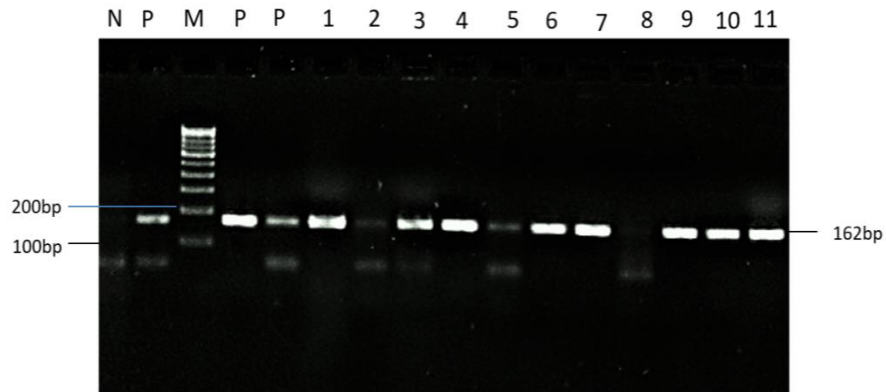
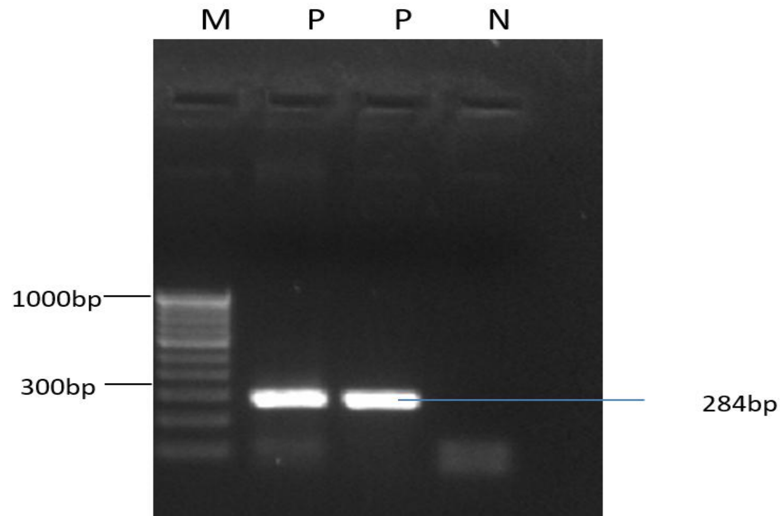


Fig.3 PCR standardization of *uid*A



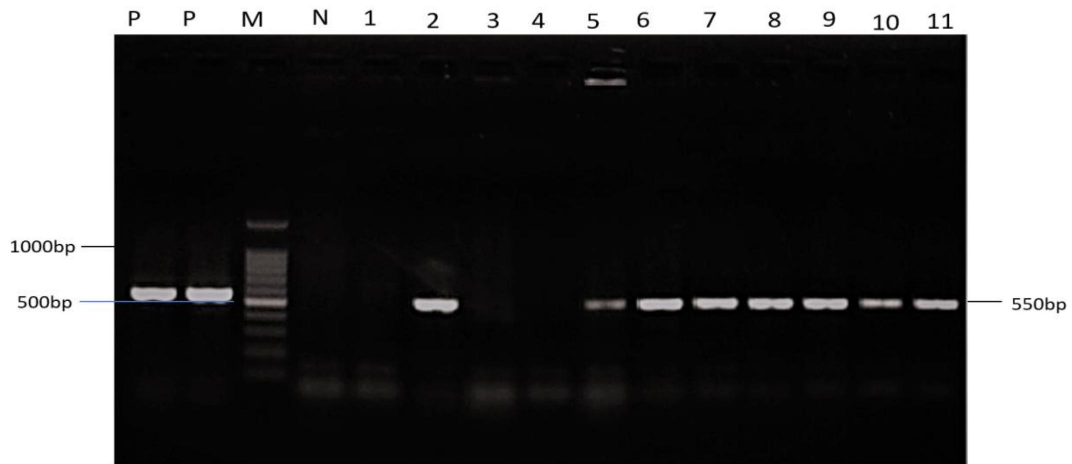
***uid*A**
Lane N- Negative control, Lane P- Positive control, Lane M- Marker, Lane 1-11: samples of amplicon size 162 bp

Fig.4 PCR standardization of *inv*A



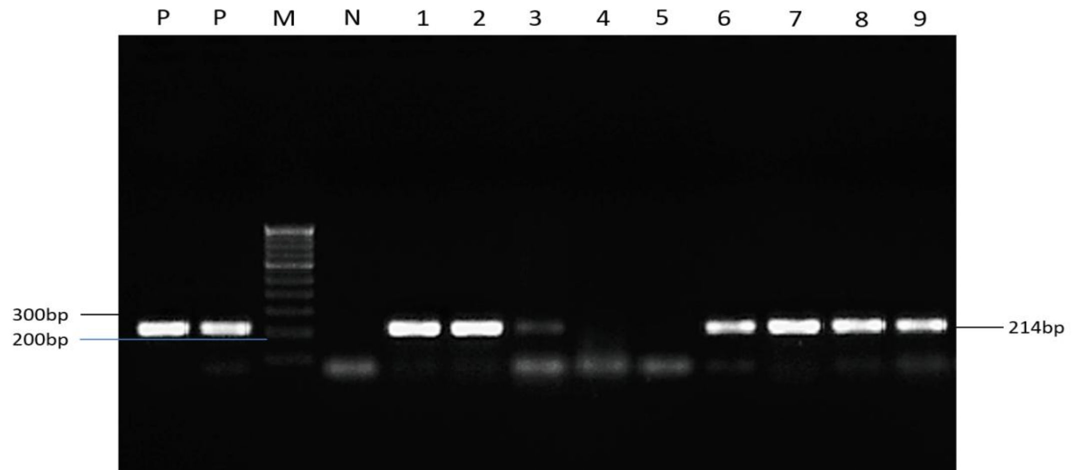
***inv*A**
Lane M- Marker, Lane P- Positive control, Lane N- Negative control.

Fig.5 PCR standardization for *bla*CTX-M



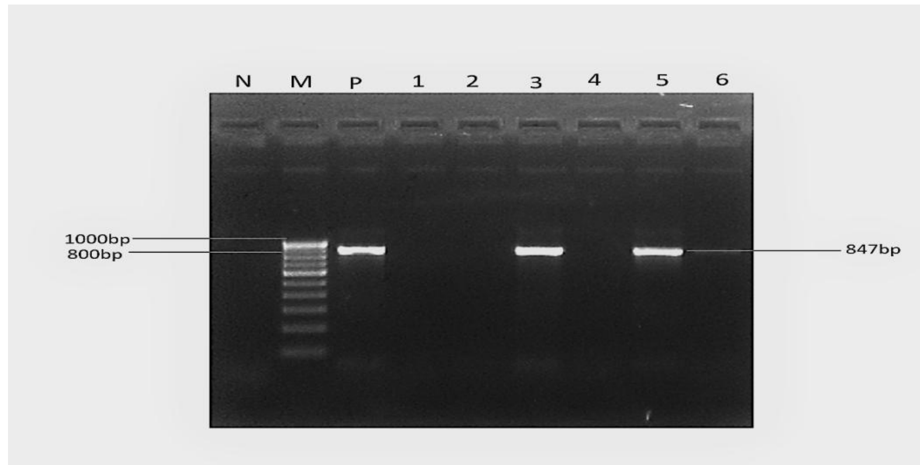
***bla*CTX-M**
Lane P- Positive control, Lane M- Marker, Lane N- Negative control
Lane 1-11: samples of amplicon size 550 bp.

Fig.6 PCR standardization for *bla*SHV



***bla*SHV**
Lane P- Positive control,
Lane M- Marker, Lane N- Negative control, Lane 1-9: samples of amplicon size 214 bp

Fig.7 PCR standardization for *bla*TEM



***bla*TEM**
Lane N- Negative control, Lane M- Marker, Lane P- Positive control,
Lane 1-6: samples of amplicon size 847 bp

One *Salmonella* isolate from raw milk sample was having all the three genes. *bla*CTX-M and *bla*SHV was together present in 3 S*almonella* isolates. In contradictory, researchers like Elumalai *et al.,* (2014) and Olesen *et al.,* (2004) found higher *bla*TEM prevalence among *Salmonella* isolates but in the present study revealed *bla*SHV and blaCTX-M was found as more prominent gene among *Salmonella* isolates. This variation in the occurrence of gene is attributed to the geographical area where study has undertaken, sample size, type of sample and drugs used and its dosage in particular area. This higher prevalence of genes among both organisms is the indication of widespread of resistant genes among human and domestic animals in Wayanad district which is a matter of great importance. Strict measures should be taken to reduce the spread of this resistant gene among human and animal communities.

Thus the present study revealed the significant occurrence of ESBL producing *E. coli* and *Salmonella* spp. from raw milk samples. Presences of antibiotic resistance to third generation cephalosporins were detected among the organisms which can transmit to humans through milk. Resistant organisms in raw milk indicate indiscriminate use of antibiotics among food producing animals. Strict legislative measures have to be taken against this and to prevent further transmission of resistant pathogenic organisms to humans.

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