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ORIGINAL RESEARCH

Prevalence, genetic profile of virulence determinants and multidrug resistance of *Escherichia coli* isolates from foods of animal origin

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Abstract

Aim: The aim of this study was to assess the hygienic quality of foods of animal origin. Thus samples from foods of animal origin, viz. mutton, chicken meat, milk and milk products were processed.

Materials and Methods: Two hundred samples from foods of animal origin viz., mutton, chicken meat, milk and milk products were processed for isolation of *Escherichia coli*. The isolates were got serotyped and also subjected to detection of virulence genes viz., *stx1, stx2, eaeA* and *hlyA* by PCR.The isolates were also tested against commonly used antibiotics.

Results: The prevalence of *E. coli* was 30% in mutton, 40% in chicken meat, 33.96% in milk and14.89% in milk products samples. All the 60 isolates of *E. coli* were grouped into 24 serogroups with O60 and O123dominant strains (8.33%) followed by O22 (6.66%). The PCR detected 21(10.5%) of samples possessing *stx1*, 14(7%) *stx2*, 3(1.5%) both *stx1* and *stx2*, 16(8%), *eaeA* and 4(2%) *EHEC-hlyA* gene. However, the prevalence of Shiga toxin producing *E. coli* (STEC) was 20% in mutton, 30% in chicken meat, 16.98% in milk and 8.51% in milk products. Whereas the prevalence of enteropathogenic *E. coli* (EPEC) was 2%, in mutton, 4% in chicken meat, 7.54% in milk and 2.12% in milk products samples. The 4 isolates O60, O101, O131 and one untypeable strain possessed the *EHEC-hlyA* gene. 22 of 50 (44%) of isolates from meat, milk and milk products showed multidrug resistance to four or more antimicrobial comprising ten of 25 (40%) isolates from chicken meat samples and 12 of 25(48%) from milk and milk products were multidrug resistance to four or more antimicrobial.

Conclusions: It is concluded that partial cooked or raw milk, meat and their products prepared under unhygienic conditions may not be directly consumed as they may be carrying the pathogenic microbes.

Keywords: drug resistance, food, prevalence, serogroup

Introduction

Among emerging foodborne bacterial pathogens, Shiga toxin producing *Escherichia coli* (STEC) is a pathogen of concern associated with the change in the livestock practices, food processing techniques along with change in food habits of people. The pathogen whose reservoirs include the gastrointestinal tract of animals especially ruminants viz., cattle, sheep and goats is mainly transmitted to humans through oral route. The oral route of transmission is of significance as various food products viz., meat, milk and their products derived from animals can be contaminated by intestinal contents of animals during production and ingestion of inappropriate processed foods and could lead to serious complications including haemorrhagic colitis (HC) or the haemolytic uraemic syndrome (HUS) in children [1,2].

In general, the pathogenicity of STEC is governed by two phage - encoded cytotoxins called shiga toxins viz., Stx1 and Stx2,produced by *stx1* and *stx2* genes, respectively [3]. In addition to these toxins,the presence of *eaeA* gene encoding 'intimin' protein enhances the virulence of STEC causing intimate attachment to the intestinal epithelial cells [4]. Also, *EHEC-hlyA* gene encoding enterohaemolysin has synergistic effect on virulence [5].

Thus the aim of this study was to elucidate the presence of *E.coli* with emphasis on STEC and their virulence determinants in food of animal origin of Jammu region.

Materials and Methods

A total of 200 samples from foods of animal origin viz.mutton, chicken meat, milk, and milk products (ice-cream, kulfi, paneer, milk cake, rasmalai, cream roll) were collected (Table-1) from Jammu region and processed as per the standard microbiological techniques [6]. The isolation of *E.coli* was achieved by enrichment in selective *E. coli* broth and plating on MacConkey agar (MA). 3-4 lactose fermenting colonies from MA were selected and streaked on EMB agar. The colonies producing metallic sheen were selected for biochemical identification.

Serogrouping: All the *E. coli* isolates were serotyped from National Salmonella and Escherichia Centre, Central Research Institute, Kasauli-173204 (H.P) India.

Polymerase Chain Reaction (PCR) for detection of *stx1, stx2, eaeA and EHEC-hlyA* genes: Primers used in the study are listed in Table 2. The template DNA was prepared as per the method of Blanco *et al*. [8] with slight modifications. The *E. coli* isolates were cultured

Table-1. Distribution of samples collected

Sr.No.	Types of sample	No. of samples
1.	Mutton	50
2.	Chicken	50
3.	Milk	53
4.	Milk products	47
Total		**200**

Table-2. List of oligonucleotide primers*(5-3) used for detection of *stx1*, *stx2*, *eaeA*, and *EHEC-hlyA* gene

Primer	Sequence (5'–3')*	Amplicon size	Reference
Stx1-F	CAACACTGGATGATCTCAG	350 bp	[7]
Stx1-R	CCCCCTCAACTGCTAATA		
Stx2-F	CTTCGGTATCCTATTCCCGG	478 bp	[8]
Stx2-R	GGATGCATCTCTGGTCATTG		
EaeA-F	GACCCGGCACAAGCATAAGC	384bp	[3]
EaeA-R	CCACCTGCAGCAACAAGAGG		
HlyA-F	GCATCATCAAGCGTACGTTCC	534bp	[3]
HlyA-R	AATGAGCCAAGCTGGTTAAGCT		

Table-3. Prevalence of *E. coli* in foods of animal origin

S.No.	Types of samples	Samples analyzed	Positive for *E. coli*	Prevalence of *E. coli* (%)
1.	Mutton	50	15	30.0
2.	Chicken	50	20	40.0
3.	Milk	53	18	33.96
4.	Milk products	47	07	14.89
	Total	**200**	**60**	**30.0**

in brain heart infusion broth at 37°C for 4 hours. One ml of the broth culture was centrifuged at 8000 rpm for 5 minutes followed by washing of pellet with NSS at 8000 rpm for 5 minutes. The pellet was mixed with 0.5 ml of nuclease free water and subjected to heat lysis in boiling water bath for10 minutes followed by immediate cooling at -20°C for 10 minutes. The mixture was centrifuged at 400 rpm for 4 minutes. 2.5µl of supernatant was used as template for PCR.

For *stx* genes duplex PCR as per the method of Paton and Paton, [3] with suitable modifications was employed using following reaction mixture components:

10X Taq buffer (with 1.5mM $MgCl_2$) = 2.5µl, Deoxynucleotide triphosphate (dNTP) 200µM each= 2.5µl, $MgCl_2$ (25mM)= 2.5µl, Primers 10 pmol each= 1.0µl, Taq DNA polymerase 1U= 0.5µl, Template DNA = 2.5µl, Nuclease free water up to= 25µl

PCR was performed in eppendorf gradient thermo-cycler with heated lid using cycling conditions as follows: initial denaturation at 94°C for 4 minutes, 35 amplification cycles each of 1 minute denaturation at 94°C, annealing at 55°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. The PCR product was analyzed byagarose gel electrophoresis. The PCR for *eaeA* and EHEC-*hlyA* genes was performed as per the method of [3] with minor modifications. For both genes, the cyclic conditions were different while the reaction mixture components were same. The components for reaction mixture were: 10X Taq buffer (with 1.5mM $MgCl_2$) = 2.5µl, Deoxynucleotide triphosphate (dNTP) 200µM each= 2.5µl, $MgCl_2$ (25mM) = 2.5µl, Primers 10 pmol each= 1.0µl, Taq DNA polymerase 1U= 0.5µl, Template DNA= 2.5µl, Nuclease free water up to= 25µl

The cycling parameters used were initial denaturation at 94°C for 5 min followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute followed by final extension at 72°C for 1 minute; while for *eaeA* gene amplification, the thermocycler conditions were same as those of *hlyA* except that the annealing temperature used for *eaeA* was 63°C for 1 minute.

The amplified products were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide @ 0.5µg/ml along with 100 bp molecular weight DNA marker (Bangalore Genei)in horizontal electrophoresis unit (Biometra, Germany) for 2 hours at 7 v/cm. The gel was visualized under UV transilluminator (Biometra, Germany).

Antibiotic sensitivity/Resistance pattern of *E.coli* isolates: A total of 50 isolates (25 from mutton and chicken meat and 25 from milk and milk products) were subjected to disc diffusion antibiotic sensitivity testagainst 15 commonly used antibiotics (Table-6)[9]. The results were interpreted as per supplier's instructions.

Results

Prevalence of *E. coli* in foods of animal origin: On testing 200 samples of foods of animal origin, the overall prevalence of *E. coli* was 30 % (60) including 30% mutton, 40% chicken, 33.96% milk and 14.89% milk products positive for *E. coli* (Table-3).

Serogroups of *E. coli* isolates: The 60 *E. coli* isolates belonged to 24 different serogroups with 5 rough and 18 untypeable strains. The detailed results of *E. coli* serogroups of each category of food are shown in Table-4.

Detection of *stx1*,*stx2*, *eaeA* and *EHEC-hlyA* genes: Screening of samples for the presence of *stx1*, *stx2*, *eaeA* and *EHEC-hlyA* gene was done by PCR (Fig.1 & 2). Sixty*E. coli* isolates comprising of 15from mutton, 20 from chicken meat, 18 from milk, and 7 from milk products were examined for the presence of these genes. 38(19%) of samples possessed *stx1*and/or *stx2* genes and were designated as STEC. PCR revealed that 21 (10.5%) samples harboured only *stx1* gene, 14 (7%) only *stx2* and 3 (1.5%) both *stx1*and *stx2* genes; whereas 8(4%) possessed the *eaeA* geneonly but not *stx* designated as Enteropathogenic *E. coli* (EPEC) (Table-5). Only 4(2%) samples possessed *EHEC-hlyA* gene belonging to serogroup O60, O101, O131 and one untypeable strain.

Prevalence of (STEC) and enteropathogenic *E. coli* (EPEC) among mutton, chicken meat, milk and milk product samples: Prevalence of STEC and EPEC from mutton, chicken meat, milk and milk products samples is presented in table 5. 20% mutton samples yielded STEC belonging to serogroup O60(4), O80, O22(2), O102, with one rough, and one untypeable whereas 2% belonging to serogroup O123were EPEC. 30% samples carrying *E. coli* strains belonging to seven sergroups (O5, O8, O20, O22(2), O102, O147, O162,

Table-4. Distribution of *E. coli* serogroups in foods of animal origin

Types of Sample Analysed	No. of *E. coli* isolates	Serogroups[#]
Mutton	15	O22(2), O60(4), O80(1), O102(1), O123(5),R*(1), UT* (1)
Chicken	20	O5(1), O8(1), O20(1), O22(2), O102(1), O147(1), O162(1), R*(2), UT* (10).
Milk	18	O12(1), O15(1), O23(1), O36(1), O86(1), O95(1), O100(1), O101(1), O109(1), O117(1), O131(1), O156(1), O164(1), R*(2), UT* (3).
Milk products	07	O60 (1), O82 (1), O144 (1), UT*(4).
Total	60	

*R=rough, UT= untypeable, [#]figure in parenthesis indicates number of isolates

Table-5. Genetic profile and prevalence of Shiga toxin-producing *E. coli* (STEC) and Enteropathogenic *E. coli* (EPEC) from foods of animal origin*

Types of samples analysed	No. of samples analysed	Prevalence *stx1*	*stx2*	*stx1& stx2*	*EHEC-hlyA*	*EPEC(eaeA)*	*STEC*
Mutton	50	5(10)	4(8)	1(2)	1(2.0)	1(2.0)	10(20.0)
Chicken	50	9(18)	5(10)	1(2)	1(2.0)	2(4.0)	15(30.0)
Milk	53	5(9.43)	3(5.66)	1(1.88)	2(3.77)	4(7.54)	9(16.98)
M. products	47	2(4.25)	2(4.25)	0(0)	-	1(2.12)	4(8.51)
Total	200	21(10.5)	14(7)	3(1.5)	4(2.0)	8(4)	38(19.0)

* figure in parenthesis indicates number of isolates

Table-6. Antimicrobial sensitivity/resistance pattern of *E. coli* isolates from meat, milk and milk products

Sr.No.	Antimicrobial agents	No. of isolates from Meat (n=25)			No. of isolates from Milk & Milk products (n=25)		
		S*	I*	R*	S*	I*	R*
1	Amikacin (Ak), 30µg	19(76)	0	6(24)	18(72)	2(8)	5(20)
2	Ampicillin, (A), 10 µg 8(32)	1(4)	16(64)	9(36)	2(8)	14(56)	
3	Amoxicillin, (Am), 30µg9(36)	2(8)	14(56)	8(32)	3(12)	14(56)	
4	Cephotaxime (Ce), 30µg	13(52)	2(8)	10(40)	12(48)	2(8)	11(44)
5	Chloramphenicol(C) 30µg	20(80)	5(20)	0	19(76)	3(12)	3(12)
6	Ciprofloxacin (Cf) 5µg 15(60)	2(8)	8(32)	15(60)	0	10(40)	
7	Co-trimoxazole(Co) 25µg	16(64)	4(16)	5(20)	15(60)	5(20)	5(20)
8	Colistin, (Cl) 10 µg 16(64)	1(4)	8(32)	15(60)	0	10(40)	
9	Cephoxitin (Cn) 30µg 21(84)	2(8)	2(8)	20(80)	2(8)	3(12)	
10	Gentamicin (G), 10 µg 20(80)	2(8)	3(12)	18(72)	2(8)	5(20)	
11	Nalidixic acid (Na) 30µg	14(56)	3(12)	8(32)	15(60)	2(8)	8(32)
12	Norfloxacin (Nx), 10 µg21(84)	3(12)	1(4)	20(80)	3(12)	2(8)	
13	Tetracycline, (T), 30µg 20(80)	2(8)	3(12)	19(76)	3(12)	3(12)	
14	Tobramycin (Tb), 10 µg	19(76)	3(12)	3(12)	18(72)	0	7(28)
15	Streptomycin (S), 10 µg	19(76)	2(8)	4(16)	18(72)	2(8)	5(20)

S = Sensitive, R = Resistant, I = Intermediate, n=number of the isolates tested, figure in parenthesis indicates number of isolates

with two rough, and 5 untypeable from chicken meat were STEC and 4% were EPEC from chicken meat carrying *E. coli* isolates, one rough and one untypeable. The prevalence of the STEC and EPEC was 16.98% and 7.54% from milk, 4.25% and 2.12% that of milk products samples respectively.

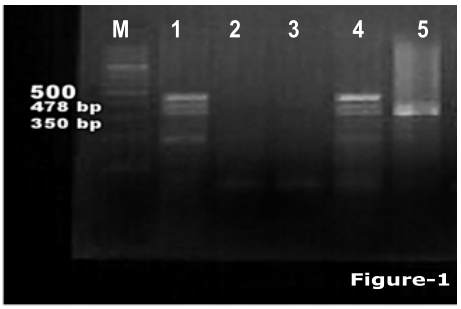
Antimicrobial sensitivity/resistance pattern of *E.coli* isolates from meat: Out of 35 isolates from meat (mutton and chicken), 25 were tested against various antibiotics for sensitivity/resistance pattern. It was found that 10 of 25(40%) isolates revealed multidrug resistance to four or more antibiotics. 76% of isolates were sensitive to Amikacin, Streptomycin and Tobramycin, 80% to Chloramphenicol, Gentamicin and Tetracycline, 84% to Norfloxacin and Cephoxitin, 60% to Ciprofloxacin, 64% to Colistin and Co-trimoxazole, and 32% to Ampicillin, 36% to Amoxycillin, 52% to Cephotaxime and 56% to Nalidixic acid. Whereas 24% isolates showed resistant to Amikacin, 64% to Ampicillin, and 56% to Amoxycillin 40% to Cephotaxime, 32% to Nalidixic acid, Ciprofloxacin, and Colistin, 20% to Co-trimoxazole, 12% to Tetracycline, Tobramycin, Gentamicin and16% to Streptomycin. Where, as 20% intermediate sensitivity was shown against Chloramphenicol (Table 6).

Antimicrobial sensitivity/resistance pattern of *E. coli* isolates from milk and milk products: Twelve of 25(48%) isolates from milk and milk products showed multidrug resistance to four and more antibiotics. But 80% were sensitive to Norfloxacin and Cephoxitin. 76% to Tetracycline and Chloramphenicol, 72% to Amikacin, Gentamicin, Tobramycin, and Streptomycin, 60% to Ciprofloxacin, Co-trimoxazole, Colistin, and Nalidixic acid, where as 56% of isolates were resistant to Amoxycillin, and Ampicillin, 44% to Cephotaxime, 40% to Ciprofloxacin and colistin, 20% to Amikacin, Co-trimoxazole, Gentamicin, and Streptomycin, 28% to Tobramycin, 12% Tetracycline. 32% to Nalidixic acid (Table 6).

Discussion

Due to ability of STEC to cause outbreaks of fatal illness it has attracted worldwide attention and many studies on STEC have been conducted in different parts of the globe.

It was observed that 60 of 200 (30%) samples were contaminated with *E. coli* out of which 19% were possessing STEC. However, the prevalence of STEC was 13 % in milk and milk product samples and 25% in meat (mutton and chicken) samples. Vernozy-Rozand, *et.al.* [10] reported 13% STEC in raw milk products samples and Kiranmayi and Krishnaiah [11] detected 24% of STEC in mutton and chicken meat samples



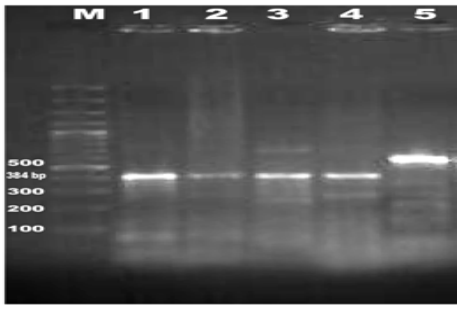


Fig.-1. Agarose gel showing the amplification product of PCR performed on *E. coli* strains for *stx1*and *stx2*genes. Lane M: 100 bp molecular weight marker. Lane 1: Positive control of 350 bp of *stx1* and 478 bp of *stx2*gene, Lane 2: Negative control, Lane 3: Negative sample, Lane 4: Amplified product of 350 bp of *stx1* and478 bp of *stx2*gene, Lane 5: Amplified product of 350 bp of *stx1* gene

Fig.-2. Agarose gel showing the amplification product of PCR Performed on *E. coli* strains for *eaeA* and *EHEC-hly* genes, Lane M: 100 bp molecular weight marker, Lane 1, 2, 3, 4: Amplified product of 384 bp of *eaeA* gene, Lane 5: Amplified product of 534 bp of *EHEC-hlyA* gene

which is in agreement to this study.

The 50 isolates from mutton, chicken meat, milk and milk products were tested against 15 commonly used antimicrobial for the treatment of food animals in different ailments. It was observed that 22(44%) of isolates were showing multidrug resistance against to four or more antimicrobials which is in agreement to the study of Altalhi, *et al.* [12], where the multidrug resistance was 40.54% among the isolates of *E. col* from chicken meat. However, 84% of isolates were sensitive to Cephoxitin, 80% to Chloramphenicol and Tetracycline from the chicken and mutton meat samples whereas, 76% to Chloramphenicol, from milk and milk product samples.

Antimicrobial resistance in *Enterobacteriaceae* poses a critical public health threat, especially in the developing countries [13,14]. Much of the problem has been shown to be due to the presence ofTransferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species [15].

Conclusion

The direct consumption of raw milk meat and their products or raw meat and partially cooked or prepared under unhygienic conditions remain high risk as they may be carrying STEC. More research in this behalf is needed to know the presence of STEC in raw milk, meat and their products to conclude survival rate and heat tolerance during processing and also to know the possibility of post preparation contamination of products.

Author's contribution

MR collected and processed the samples, drafted the manuscript. SKK and MAM revised the manuscript MS helped in design, online submission and necessary suggestions. All author read and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interest.

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