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Research Article
Virulence Attributes and Antibiotic Resistance Pattern of *E. coli* Isolated from Human and Animals

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Abstract
Esherichia coli is one of the most important zoonotic enteric pathogen and most widely accepted indicator of fecal contamination in food and water. In present investigation, *E. coli* was isolated and identified in the faecal samples of pig, cattle and the farm workers handling the animals (20 each) using conventional and molecular methods and their virulence attributes and antibiogram were investigated. Total 40 isolates were tentatively identified as *E. coli* by conventional method while *uspA* gene was detected in only 17 (31%) isolates. A total of 46% isolates appeared pathogenic base on the virulence traits viz., haemolytic assay, congo red binding, haemagglutination, mannose resistant and mannose sensitive haemagglutination. In antibiogram study, all human and cattle isolates exhibited resistance to kanamycin, ampicillin, penicilline-G, cephalaxin, neomycin, streptomycin and ofloxacin while all cattle isolates were also found resistant to gentamycin and doxicycline. All the isolates from pigs exhibited resistance for gentamycin, kanamycin, penicillin-G, cephalexin and ampicilllin. Most important finding was the multiple drug resistance exhibited by majority of isolates involving as much as 5 antibiotics, which is an alarming situation posing threat to human and animal health.

Key words: *Esherichia coli*, virulence attributes, *Usp*A gene, congo red binding, MRHA, MSHA, antibiogram

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INTRODUCTION

Esherichia coli bacteria are essential to healthy functioning of human and animal digestive system. For many years, the bacterium was simply considered as commensal organism of the large intestine. It was until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants (Osman *et al*., 2012). The emergence of verotoxic *E. coli* O157:H7, the cause of the two unique outbreaks in 1982 in Oregon and Michigan, USA, further outlined the importance of *E. coli* (Riley *et al*., 1983). Subsequently, several serotypes of verotoxin producing *E. coli* (VTEC) have been reported from almost all the countries. *Esherichia coli* has again become a focus after a highly publicized latest outbreak that occurred in Germany and other European countries in which vegetables were incriminate as source of infection (Stevens and Hobson, 2011). There are many evidences that food can be reservoir of drug-resistant *E. coli* causing extra intestinal infections (Vincent *et al*., 2010; Manges and Johnson, 2012; Dutta *et al*., 2014).

Emergence of resistance is almost certainly an inevitable consequence of indiscriminate clinical use of antimicrobial drugs for treating human and animal diseases and their sub-therapeutic use for disease prevention and growth promotion in livestock and poultry (Landers *et al*., 2012). Antimicrobial resistance of *E. coli* gives a special alarm, because more than 150 *E. coli* serotypes are reportedly shared by human being and animals (Ferens and Hovde, 2011) and some serotypes have picked up "Pathogenicity islands" that can turn a harmless bacterium into pathogenic one (Rangel *et al*., 2005). Therefore, present study was designed to isolate *E. coli* from both farm animals and farm workers to study their virulence characteristics and antibiogram.

MATERIALS AND METHODS

Samples: A total of 60 faecal samples comprising 20 each from cattle, pigs and human (Farm workers) were collected aseptically in sterilized test tubes by swab technique and brought to the laboratory on ice. In addition, 14 serotyped *E. coli* of poultry (O4, O5, O11, O24, O32, O34, O60, O65, O69, O83, O148, R, UT(2)) isolated and maintained in the department were included in molecular and virulence study.

Isolation and identification: *Esherichia coli* were isolated using the method of Cruickshank *et al.* (1975). Briefly, the samples were inoculated in 2 mL of nutrient broth and incubated aerobically for 12 h at 37°C. Thereafter, the cultures were streaked on to MacConkey Lactose Agar (MLA) followed by Eosin Methylene Blue (EMB) agar plates and incubated at 37°C for 24 h. The colonies exhibiting rose pink colour on MLA and metallic sheen on EMB plates were transferred to nutrient agar slants and stored at 4°C until processed further. The isolates were subjected to Gram's staining and motility test followed by biochemical characterization viz., IMViC pattern, catalase, oxidase, Triple sugar iron tests and fermentation of glucose, lactose, sucrose, fructose, maltose, mannitol and sorbitol sugars. The identified *E. coli* isolates were serotyped at Central *Escherichia* and *Salmonella* centre, Kasauli, Himanchal Pradesh, India.

Molecular identification: The isolates identified as *E. coli*, were further tested for presence of *uspA* gene by polymerase chain reaction using published primer sequence F-CCGATACGCTGCCAATCAGT and R-ACGCAGACC GTAGGCCAGAT (Anastasi *et al*., 2010) synthesized by Bangalore Genei (India).

The DNA templates were prepared using snap-chill method (Franco *et al*., 2008). The PCR was performed in final volume of 25 µL containing 12.5 µL of 2x master mix, 2 µL (10 pmol) of forward and reverse primer, 2 µL of DNA template and nuclease free water 6.5 µL. The PCR cycling condition included initial denaturation at 95°C for 5 min followed by 30 cycles at 94°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 30 sec and final extension at 72°C for 5 min. The amplified PCR products were electrophoresed in 1.5% agarose gel and visualized under gel documentation system (Uvi tech, UK).

In vitro study for virulence traits

Haemolysin assay: *Esherichia coli* isolates were tested for haemolysin production by plate as well as tube inoculation method. In plate method, 5% w/v sheep blood agar plates supplemented with 10 mM calcium chloride were inoculated and incubated overnight at 37°C as per the method of Beutin *et al.* (1996). Haemolysin production was indicated by the appearance of zone of complete lysis of erythrocyte. The tube method was used for alpha-haemolysin production. Isolates were inoculated in 1 mL of sterilized nutrient broth supplemented with equal volume of washed sheep RBCs (5% V/V) in graduated centrifuge tube. The tubes were incubated at 37°C for 4-6 h with intermittent agitation of the culture followed by centrifugation at 6000 rpm for 5 min. Transparent reddish color of supernatant, sign of haemolysis, indicated haemolysin production.

Mannose resistant haemagglutination (MRHA) and mannose sensitive haemagglutination (MSHA): The MRHA and MSHA property of *E. coli* isolates was determined by using mannose sensitized 5% sheep RBCs as described by Green and Thomas (1981) with slight modification. The isolates sub cultured in nutrient broth were centrifuged at 10,000 rpm for 10 min and pellet was washed in phosphate buffer saline. Before testing, cell concentration was adjusted to approximately 2×10^{10} CFU mL^{-1} by the McFarland turbidimetric method and sheep RBCs suspension was mixed with equal amount of 2% D (+) mannose solution and kept for few min at 4°C.

Fifty microliter of bacterial suspension was emulsified in equal amount of PBS at two spots on a microscopic slide. Fifty microliter of 5% RBC suspension without mannose was then added on one spot and 50 µL of RBCs suspension with mannose on the other spot. The contents were mixed thoroughly by rotating the slide gently in circular manner. The clumping of RBCs without mannose side was considered as positive for haemagglutination. On the opposite side, presence of clumping of RBCs treated with mannose was assumed as MRHA while absence of clumping was considered as MSHA. The suspension of RBCs with and without mannose in PBS was taken as negative control.

Congo red binding assay: The Congo Red test (CR test) was carried out as per the technique of Berkhoff and Vinal (1986). The isolates were streaked on the plate of Trypticase Soya Agar (TSA) containing 0.03% congo red dye and incubated at 37°C for 24-72 h. Appearance of brick red colonies within 24-72 h was considered as positive while the colonies that remained white or grey even after 72 h PI were regarded as negative.

Antibiogram study: The *E. coli* isolates recovered from cattle, pig and human were subjected to antibiogram study against 15 different antibiotics using the modified disc diffusion method of Bauer *et al.* (1966). The isolates were tested against ampicilline (Amp), chloremphenicol (C), cephalaxin (Cp), ciprofloxacin (Cf), cephataxim (Ce), doxycline (Do), furazolidone (Fr), gentamycin (Gn), kenyamycin (K), Neomycin (Ne), norfloxacin (Nor), ofloxacine (Of), penicilline G (P), streptomycine (S) and tetracycline (TE).

RESULTS

Isolation and identification of *E. coli* from faecal samples was attempted by conventional technique followed by polymerase chain reaction with an aim to develop a rapid and sensitive method for detection of *E. coli*. Processing of 60 samples yielded 40 isolates that showed typical characteristics of *E. coli* on MLA and EMB media and Gram's staining (G-ve coccobacilli). Biochemically, IMViC pattern (++--), acid and gas production on TSI also revealed typical characteristic of *E. coli*. All the isolates fermented fructose, maltose, lactose, glucose and mannitol sugars with production of either acid or acid and gas both while fermentation of sucrose and sorbitol was variable. Based on colony and morphological characteristics and biochemical profile, 40 isolates were identified as *E. coli*, showing over all isolation rate of 66.67%. On serotyping, these isolates belonged to O:1 (3), O:2 (3), O:9 (3), O:26 (3), O:59 (4), O:156 (8) and untypable (16). These 40 isolates and 14 serotyped *E. coli* of poultry were further subjected to PCR in which, 17 isolates harbored *UspA* gene with an amplicon size of 884 bp.

The isolates were further tested for pathogenic traits viz., haemolytic assay, haemagglutination test and Congo red binding test. Haemolytic assay exhibited haemolysis in total 19 (35%) isolates of which 15 (27%) were positive in plate method and all the 19 (35%) isolates were positive in tube method. The highest isolates showing haemolytic ability belonged to human (44.4%) followed by cattle (42.8%), pig (35.2%) and poultry (21.4). Haemagglutinating ability was revealed by 22 (40%) isolates, of which 14 (25%) isolates showed MRHA and 8 (14%) isolates showed MSHA. Highest percentage of MRHA ability was seen in cattle isolates (42.8%) followed by pig (23.5%), human (22.2%) and poultry (14.2%), whereas the highest isolates exhibiting MSHA property were from human (22.2%) followed by pig (17.6%), cattle (14.2%) and poultry (7.1%). In Congo red binding assay, 36 (66%) isolates produced brick red colonies and considered as enteropathogenic. Maximum CR positives isolates were from pig (88.2%) followed by poultry (71.4%), cattle (50.0%) and human (44.4%).

Among isolates from human beings, antibiogram of *E. coli* isolates revealed chloramphenicol (100%), ciprofloxacin (100%) and gentamycin (77%) to be the most effective antibiotics while furazolidon (100%) and norfloxacin (44%) were found moderately effective and all isolates appeared completely resistant for kanamycin, ampicillin, penicilline G, cephalaxin, neomycin, streptomycin and ofloxacin (Table 1). In cattle isolates, most effective antimicrobial agents were found to be chloramphenicol (100%) and ciprofloxacin (92%) while tetracycline (50%), norfloxacin and furazolidone (21%) were found moderately effective (Table 1). All the cattle isolates were resistant for gentamycin, kanamycin, ampicillin, penicillin G, cephalaxin, ofloxacin while low percentage of isolates were resistant to norfloxacine and furazolidone (57.1% each), tetracycline (50.0%) and ciprofloxacin (28.5%).

Table 1: Antibiotic susceptibility of *Esherichia coli* isolates of human, cattle and pig origin

		No. of human isolates (%)			No. of cattle isolates (%)			No. of pig isolates (%)		
Antibiotic	Conc. (µg/disc)	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Chloramphenicol	30	0	0	9 (100)	0	0	14 (100)	1 (5.8)	1 (5.8)	15 (88.2)
Gentamycin	120	0	2 (22.2)	7 (77.7)	14 (100)	0	0	17 (100)	0	0
Norfloxacin	10	4 (44.4)	4 (44.4)	1 (11.1)	8 (57.1)	3 (21.4)	3 (21.4)	3 (17.6)	13 (76.4)	1 (5.8)
Kanamycin	30	9 (100)	0	0	14 (100)	0	0	17 (100)	0	0
Furazolidone	50	0	9 (100)	0	8 (57.1)	3 (21.4)	3 (21.3)	0	13 (76.4)	4 (23.5)
Ampiciliin	10	9 (100)	0	0	14 (100)	0	0	17 (100)	0	0
Tetracycline	10	7 (77.7)	2 (22.2)	0	7 (50.0)	7 (50.0)	0	8 (47.0)	7 (41.1)	0
Penicillin G	30	9 (100)	0	0	14 (100)	0	0	17 (100)	0	0
Cephalexin	30	9 (100)	0	0	14 (100)	0	0	17 (100)	0	0
Ciprofloxacin	5	0	0	9 (100)	4 (28.5)	0	10 (71.4)	0	3 (17.6)	14 (82.3)
Cefataxime	30	6 (66.6)	1 (11.1)	2 (22.2)	10 (71.4)	4 (28.5)	0	5 (29.4)	7 (41.1)	5 (29.4)
Doxycycline	30	5 (55.5)	0	4 (44.4)	14 (100)	0	0	10 (58.8)	7 (41.1)	0
Neomycin	30	9 (100)	0	0	14 (100)	0	0	1 (5.8)	7 (41.1)	9 (52.9)
Streptomycin	10	9 (100)	0	0	14 (100)	0	0	4 (23.5)	4 (23.5)	9 (52.9)
Ofloxacin	2	9 (100)	0	0	14 (100)	0	0	6 (35.2)	6 (35.2)	5 (29.4)

In pigs, the best antibiotics in terms of sensitivity were found to be chloramphenicol (88%) and ciprofloxacin (82%) followed by neomycin and streptomycin (52.9% each), cefataxime and ofloxacin (29.4% each). Moderately effective antibiotics were norfloxacin (76.4%), furazolidon (76.4%) and tetracycline (41.1%) while all the isolates were resistant to gentamycin, kanamycin, ampicillin, penecillin G and cephalaxin.

Multiple Drug Resistance (MDR) has been a consistent finding in the *E. coli* isolates from every species in the present study. Maximum number of the isolates exhibited resistance against more than 2 antibiotics and the phenotype Ce-CN-K-Amp-T-P-CP was predominant (8 isolates) followed by N-S-Of-K-Amp-T-P-CP (6 isolates).

DISCUSSION

Esherichia coli is a widely used indicator of fecal contamination in food and water. External contact and subsequent ingestion of bacteria from fecal contamination can cause detrimental health effects (Money *et al*., 2009). In recent years, *E. coli* has gained public health significance due to its association with life threatening human diseases like HC, HUS, TTP syndromes. Foods of animal origin are one of the important routes for the disease transmission from animals to human.

In the current study, total 40 isolates were recovered out of 60 fecal samples which belonged to O:1, O:2, O:9, O:26, O:59, O:156 serogroups and 18 isolates were untypable *E. coli*. The overall isolation rate of 66.5% was in agreement with earlier reports (Diwakar *et al*., 2014; Dutta *et al*., 2014). For molecular identification, Universal stress protein (*uspA*) gene which has been invariably used as a marker to differentiate pathogenic *E. coli* from other Gram negative Enterobacteria (Nachin *et al*., 2005) was targeted. But in present study, *uspA* gene was not consistently present in all the isolates, merely 17 out of 54 isolates harbored *uspA* gene.

Sugar fermentation among *E. coli* isolates was readily used to distinguish *E. coli* from other pathogenic fecal coliforms (Aklilu *et al*., 2013). The isolates in present study fermented fructose, maltose, lactose, glucose and mannitol but sucrose and sorbitol was not fermented by all isolates. In last decade, non-motile sorbitol fermenting isolates have emerged as important causes of human diseases and previous studies have also revealed that inability to ferment sorbitol might be used as an indicator to distinguish hemorrhagic strains of *E. coli* from non hemorrhagic strains (Regua-Mangia *et al*., 2008). To establish correlation between the two attributes, comparative analytical study was done with all the isolates. Out of 44 (81.48 %) sorbitol positive isolates, 26 (59.09%) gave haemolysis while among 10 (18.51%) sorbitol negative isolates, 7 (70.00%) were positive for haemolysis. Therefore, no such correlation could be established between haemolytic property and sorbitol fermentation. The findings are in agreement with earlier reports that the screening of *E. coli* on the basis of phenotypic characteristics such as sorbitol non-fermentation is not sufficient and for effective detection of non-motile sorbitol fermenting EHEC, screening for shiga toxins by ELISA and/or shiga toxin genes by PCR is absolutely necessary (Orth *et al*., 2009).

Haemolysin may play an important role in lyses of the endocytic vacuole to permit escape of the bacteria into the cytoplasm of colonic epithelial cells (Koli *et al*., 2011). In this study, human and cattle isolates were found to have higher haemolytic ability than pig. These workers might be suffering from asymptomatic infection of VTEC strains due to

occupational exposure. A correlation between VTEC and enterohaemolysin was reported previously by several workers (Al-Charrakh and Al-Mmuhana, 2010; Vaishnavi *et al*., 2010). In haemolytic assay, two methods were compared and more number of isolates was recorded positive by tube method than plate test, so tube method may be regarded as a more rapid and better alternative of plate method for detection of alpha haemolysin.

In the present study, agglutination of erythrocytes was revealed by 22 (40%) isolates out of 54, which is an indirect evidence of presence of fimbriae in enterotoxigenic *E. coli* (Shetty *et al*., 2014). Strains possessing fimbriae can haemagglutinate erythrocytes (RBCs) because they have receptors to fimbriae on their surfaces similar to those found on enterocytes (Shetty *et al*., 2014). Besides this, 14 (25%) isolates that showed MRHA can be considered as uropathogenic *E. coli* (Maheswari *et al*., 2013) because this property has been found more frequently in isolates of *E. coli* from human urinary tract as compared to those from normal feces (Swaroop *et al*., 2013). Bacterial surface antigen (s) mediating mannose-resistant hemagglutination of human erythrocytes and attachment to human urinary tract epithelial cells may be one factor for selecting *E. coli* from among the fecal flora which infects the urinary tract (Swaroop *et al*., 2013). Fimbriae responsible for MRHA are non type 1 and include K88, K99, colonization factor antigen I and some *E. coli* surface components of colonization factor antigen II, (Gaastra and de Graaf, 1982). However, the isolates that showed MSHA might be having type 1 fimbrae which is present in most of *E. coli* including nonpathogenic one.

Binding of congo red dye by *E. coli* is associated with the pathogenicity of the organism and can be used as a phenotypic marker to distinguish invasive and non-invasive isolates of *E. coli* particularly of poultry (Berkhoff and Vinal, 1986; Osman *et al*., 2012). In this study, out of 36 (66.6%) congo red positive isolates, maximum numbers were obtained from pig (88.2%) followed by poultry (71.4%), cattle (50%) and human (44.4%) isolates. Hence, CR test can be used as indicator test to detect enteroinvasive *E. coli* not only of poultry but also of other species as reported earlier (Ingle *et al.,* 2010; Osman *et al*., 2012). The CR binding ability has been reported to be affected by the levels of curli fibres expressed at the bacterial cell surface (Osman *et al*., 2012).

Use of antibiotics for prevention and control of bacterial infections as a whole and in *E. coli* infections in particular has always been a matter of investigation as a large number of isolates have been reported to be resistant to a group of antibiotics. In the present study, the most commonly used antibiotics kanamycin, ampiciliin, penicillin, cephalexin, neomycin, streptomycin, ofloxacin were found resistant for all the isolates of human being and cattle. Similarly, all pig isolates were also resistant for gentamycin, kanamycin, ampiciliin and cephalexin. This might be because of close association between farm worker and animals leading to sharing of antibiotic resistant isolates. However, among all, only chloramphenicol and ciprofloxacin were found drug of choice for all the species as far as sensitivity is concerned. This pattern of antibiotic resistance has also been reported by many workers (Frye *et al*., 2011; Dutta *et al*., 2014; Diwakar *et al*., 2014). Hundred percent resistance against most frequently used ofloxacin in human and cattle and gentamycin in cattle and pig is a matter of great concern.

Multiple drug resistance has been a consistent finding in the *E. coli* isolates of all the species in the present study. Maximum number of the isolates exhibited resistance against more than 2 antibiotics and the phenotype Ce-CN-K-Amp-T-P-CP was predominant in most of the MAR isolates followed by N-S-Of-K-Amp-T-P-CP. This phenomenon has been reported frequently among *E. coli* isolates in last few decades (Frye *et al*., 2011; Momtaz *et al*., 2012; Millman *et al*., 2013).

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