VIRULENCE FACTORS AND DRUG RESISTANCE OF GASTROINTESTINAL *ESCHERICHIA COLI* ISOLATED FROM DIFFERENT ANIMALS

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Abstract

Escherichia coli is one of the natural colonizers in the gastrointestinal tract of several animals ranging from terrestrial to aquatic origin. Nowadays virulence attributes of *E. coli* along with antibiotic resistance pattern is a global crisis. Therefore, it is of utmost importance to monitor the possible virulence characteristics as well as the antibiotic-resistant pattern among the *E. coli* isolates residing in the gastrointestinal tract of different animals. In this study, a total of 42 *E. coli* isolates from the gastrointestinal tract of different species like pig, broiler, chicken, fish and goat, as well as from the stool samples of human, were studied for their virulence attributes and antibiotic resistance. Based on the various virulence traits like biofilm-forming, congo red binding, proteolytic, haemolytic, and haemagglutinating activity, this study indicated that 80% of isolates possessed putative factors associated with pathogenicity. Further, these isolates were found to be the extended-spectrum β -lactamase producer and resistant to at least 5 antibiotics, which is a matter of concern since the possibility of horizontal transfer and health implications in the host as well as to human can't be ruled out.

Key words: Antibiotics, Biofilm, Congo red, *Escherichia coli*, Virulence

INTRODUCTION

Escherichia coli, a rod-shaped, Gram-negative, facultative anaerobic coliform bacterium of gamma proteobacterium of *Enterobacteriaceae*, is a natural colonizer in the gastrointestinal tract of warm-blooded animals. *E. coli* are mostly commensal gut microbiota. Despite this, it is associated with several complications in different animals and humans. Contamination of water and food with faecal bacteria remains a common and persistent problem leading to outbreaks of food- and water-borne illness, impacting public health. *E. coli* isolates can be enteroinvasive, enterotoxigenic, enteroadherent, enteropathogenic, enterohemorrhagic and diffusely adherent. Shiga-

toxigenic *E. coli* can cause severe complications like bloody diarrhea, hemorrhagic colitis, thrombocytopenia, hemolytic anemia, lethal hemolytic uremic syndrome, and acute renal failure (Jafari *et al.*, 2012; de Mello Santos *et al.*, 2020). Virulence in *E. coli* isolates is often multifactorial with several virulence factors associated with biofilm production, protease activity, gelatinase activity mannose resistant haemagglutination, cell surface hydrophobicity, and serum resistance, *etc* (Shetty *et al.*, 2014; Bhrugubalda *et al.*, 2016). Therefore it is utmost importance to determine the factor responsible for diseases for adopting suitable prophylaxis measures.

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While antibiotic therapy remains the main prophylaxis measure, in the present era of ever-increasing antibiotic resistance is a matter of deep concern. Inappropriate use of antibiotics is the main reason of the emergence and dissemination of resistance genes in animals, humans and the environment (Bryce *et al.*, 2016; Sasikala *et al.*, 2019; Odongo *et al.*, 2020). Over the years, the resistance to antibiotics like cephalosporin among members of *Enterobacteriaceae* has increased mainly due to the spreading of Extended-spectrum β -lactamase (ESBL). Further, ESBL producers carry genes for resistance to other antimicrobial agents due to their presence in the same plasmid of ESBL (Rawat and Nair, 2010), and the incidence of ESBL producing *E. coli* strains have been increasing, which becomes a limiting factor for therapeutic applications (Mathur *et al.*, 2002).

Nowadays virulent attributes along with antibiotic resistance of *E. coli* is a major concern for researchers (Thomson and Moland, 2000; Bhrugubalda *et al.*, 2016; Mohammadi-Sardo *et al.*, 2017). Several characters such as biofilm-forming, congo red binding potency, proteolytic activity (Sheikh *et al.*, 2001; Shetty *et al.*, 2014) along with haemagglutination, haemolysin and haemolytic activities are attributed to the virulence of this bacterium. It is, therefore, of utmost importance to monitor the possible virulence characteristics as well as antibiotic resistance pattern among the *E. coli* isolates from the gastrointestinal tract of different animals, which in turn will help to know the prevalence of various virulent traits as well as the antibiotic-resistant pattern among the normal microbiota of animals. So that adequate prophylaxis measures can be taken to minimise and control the pathogens. Therefore, the present investigation was carried out to study the virulence attributes, antibiotic resistance pattern, and ESBL producing ability of *E. coli* isolates obtained from the gastrointestinal tract of various animals.

MATERIALS AND METHODS

Sample collection: All the experiments were conducted following standard procedures and in compliance with the ethical guideline and clearance of North Orissa University (NOU), Odisha, India. The gastrointestinal tract of different animals like pig (*Sus domesticus*), goat (*Capra aegagrus hircus*), broiler (*Gallus gallus*), fish (Rohu, *Labeo rohita*), chicken (*Gallus gallus domesticus*) were aseptically collected and processed at the Department of Biotechnology, NOU, Odisha, India for isolation of *E. coli*. Besides, stool samples from human were also processed for isolation of *E. coli*. The isolation of gastrointestinal tract from apparently healthy animals and birds with no previous history of drugs/antibiotics treatment was conducted under the supervision of a qualified Veterinarian. The isolation of gut from the animals and birds was conducted with the approval of the institutional animal ethical committee.

Isolation and identification of *E. coli* isolates:

The gastrointestinal tract (GI) of different animals were processed for isolation of *E. coli* as per standard microbiological methods (Cowan and Steel, 1975). Briefly, the gut content was removed by squeezing the tract followed by repeated washing with phosphate-buffered saline (PBS, pH 7.2). The GI tract was then homogenized with PBS with the help of mortar and pestle. Then the suspension was diluted and plated onto Mac-Conkey agar (Hi-Media, India) plates and incubated at 37°C overnight. After incubation, lactose fermenting pink colonies were picked and streaked on Eosin methylene blue (EMB) agar (Hi-Media, India) plates and incubated at 37°C for overnight. After incubation large, blue-black colonies with a green metallic sheen were picked from EMB plates and again sub-cultured onto the same media for obtaining pure culture. Finally, the individual black centered colony was maintained in slants. Similarly, stool samples were also processed for isolation of *E. coli*. After isolation, all isolates were processed through a series of biochemical

tests and isolates were identified and characterised based on the colony morphology and biochemical characteristics (Cowan and Steele, 1975; Cruickshank *et al.*, 1975).

Virulent attributes of the *E. coli*: The biofilm-forming ability of *E. coli* isolates was done as per the procedure of Christensen *et al.* (1985). Briefly, *E. coli* isolates were grown in nutrient broth at 37°C for 18 - 24 hrs. The cultured broth was decanted and the tubes were poured with 1% crystal violet solution for 1 hr. The test tubes were then washed with distilled water after gently removing the crystal violet solution. Finally, 2 mL of 30% acetic acid was added and then the OD value was measured at 600 nm. Based on OD values, the isolates were interpreted as strong positive (OD value >1.0), positive (OD value between 0.5 and 1.0) or negative (OD value < 0.5).

The ability of *E. coli* isolates to bind congo red dye was carried out in congo red agar plate as per the procedure of Berkhoff and Vinal (1986) and the proteolytic activity of the isolates was tested in skim milk agar plates as per the method of Abd Samad *et al.* (2017). Similarly, the haemolytic activity of the *E. coli* isolates was done by using 5% blood agar plates of species like human, fish, pig, broiler, goat and chicken as per the protocol of Roy *et al.* (2006).

Haemagglutinating and haemolysin activity:

The haemagglutinating activity of the *E. coli* isolates was tested against red blood cells (RBCs) from various sources like human, fish, goat and chicken as per Shetty *et al.* (2014) with slight modifications. Briefly, the assay was conducted in 'U' shaped microtitre plates by serial two-fold dilution of 50 µL bacterial sample with PBS (pH 7.2). Then 50 µL of freshly prepared 1% RBC suspension of individual species were separately added to each well. The plates were kept 28 – 30°C for 2 hrs. After

incubation, the plates were observed for agglutination of erythrocytes. The haemagglutination titre was calculated as the reciprocal of the highest dilution of bacteria showing complete agglutination of RBCs. The haemolysin activity was conducted in a similar manner and incubation was done overnight at 4°C. After incubation, haemolysis of erythrocytes was recorded and the haemolysin titre was calculated in a similar manner.

Antibiotic resistance pattern study: The antibiotics resistance pattern of *E. coli* isolates against 20 different antibiotics such as ampicillin (10 mcg), amoxicillin (10 mcg), ceftazidime (30 mcg), cefoperazone (75 mcg), cefpodoxime (30 mcg), cefepime (30 mcg), clotrimazole (10 mcg), cefazolin (30 mcg), cefotaxime (30 mcg), cotrimoxazole (25 mcg), ceftriaxone (30 mcg), chloramphenicol (30 mcg), meropenem (10 mcg), moxifloxacin (5 mcg), nystatin (100 units), nitrofurantoin (300 mcg), norfloxacin (10 mcg), nitrofurantoin (300 mcg), penicillin (10 units) and tetracycline (30 mcg) (HiMedia, India) was performed on Mueller Hinton agar at 37°C for 24 hrs as per standard agar-disc diffusion method (Bauer *et al.*, 1966). The selection of antimicrobial drugs was based on their common use, availability and as per the recommendations of Clinical and Laboratory Standards Institute (CLSI). After incubation, the zone of inhibition was measured and interpreted as sensitive, resistant and intermediate sensitive as per CLSI recommendations (Clinical and Laboratory Standards Institute, 2014).

Extended-spectrum beta-lactamase (ESBL) detection assay:

The ESBL screening was done by the standard disc diffusion method as described in ESBL kit (HiMedia, India) containing cefotaxim and cefotaxim with clavulanic acid. After incubation, the zone of inhibition was measured and the results were interpreted as per the manufacturer's instruction.

RESULTS

In this study, a total of 42 isolates were purified from the GI tract of goat (10), chicken (11), broiler (12), pig (3) and fish (2). Similarly, 4 isolates were obtained from human stool samples. All the isolates were identified as *E. coli* phenotypically (Table 1).

Virulent attributes of *E. coli*: All the *E. coli* isolates from different sources exhibited various virulent attributes like biofilm-forming, congo red binding, proteolytic and haemolytic activity. *E. coli* isolates exhibited variable biofilm-forming ability with respect to the source of isolation (Fig. 1a). All the *E. coli* isolates from

Table 1. Biochemical characteristics of *E. coli* isolates (n=42) from different sources

Tests/Parameters	Characteristics
Gram reaction	-
Morphology	Rods
Motility	Motile
Catalase	+
Oxidase	-
Methyl red reaction	+
Voges-Proskauer reaction	-
Indole production	+
Citrate utilization	-
Oxidative/fermentative reaction	+/-
Hydrogen sulfide production	-
Gas from glucose	+
Nitrate reduction	+
Fermentation of sugars	
Glucose	+
Lactose	+
Sucrose	+
Sorbitol	+
Mannitol	+
Inositol	-
Mannose	-
Fructose	-
Maltose	-
Raffinose	-
Ribose	-

human and pig were strong biofilm formers. Similarly, 50% of the *E. coli* isolates from fish and pig were strong biofilm formers. On the other hand, the highest percent of non-biofilm forming isolates were from chicken (54.55%) followed by the goat (40%). *E. coli* isolates were found to possess congo red binding potency but the pattern of the binding potency varied among isolates. All the *E. coli* isolates from human (stool) exhibited strong congo red binding activity. The percentage of *E. coli* isolates from chicken, broiler and goat with strong dye-binding activity was 63.0, 57.0 and 40.0, respectively. *E. coli* isolates from pig and fish were positive only after 72 hrs of incubation. However, 30%, 27.27% and 21.0% of the *E. coli* isolates from goat, chicken and broiler, respectively were congo red negative (Fig. 1b). *E. coli* isolates irrespective of sources exhibited proteolytic activity but their percentage varied among the source of isolation (Fig 1c). All the *E. coli* isolates from pig and human stool exhibited proteolytic activity. The percentage of isolates from goat, broiler chicken and fish with proteolytic activity was 77.0, 83.0, 75.0 and 50.0, respectively. *E. coli* isolates from all the sources except pig were haemolytic to different erythrocytes. *E. coli* isolates from goat were found to haemolyse all the tested erythrocytes. Highest percent of *E. coli* isolates (50.0) from goat haemolysed chicken erythrocytes followed by 20% of the total isolates to haemolyse both goat and fish erythrocytes (Table 2). *E. coli* isolates from fish were found to haemolyse the erythrocytes of human (50%) and fish (50%), and non-haemolytic to broiler and goat erythrocytes. Similarly *E. coli* isolates from human and chicken were haemolytic to goat, broiler and fish erythrocytes, and non-haemolytic to human erythrocytes. *E. coli* isolates from broiler were haemolytic to the erythrocytes of human, goat and broiler erythrocytes, and non-haemolytic to fish erythrocytes.

Fig. 1(a-c) Percentage of *E. coli* isolates from different sources showing different virulent characteristics a) Biofilm forming activity, b) Congored binding activity, c) Proteolytic activity

Table 2. Percentage of *E. coli* isolates of different sources showing haemolytic activity against homologous and heterologous erythrocytes

Source of <i>E. coli</i>	No. of isolates	Erythrocytes of					
		Human	Pig	Goat	Fish	Broiler	Chicken
Fish	2	50	0	0	50	0	0
Pig	3	0	33.33	33.33	0	33.33	33.33
Human	4	0	0	50	50	75	0
Chicken	11	0	0	45.45	36.36	54.54	45.45
Goat	10	10	20	20	20	10	50
Broiler	12	50	0	66.66	0	75	0

Haemagglutination and haemolysin titre: All the *E. coli* isolates were found to agglutinate erythrocytes of different species. The haemagglutinating activity of *E. coli* isolates from the pig was the least against all the tested erythrocytes. However, *E. coli* isolates from goat exhibited high haemagglutinating activity with geometric mean titre value of 1.31, 2.0, 1.0, 1.0 and 2.0 against erythrocytes of fish, broiler, pig, human and goat, respectively (Table 3). Similarly, all the *E. coli* isolates were haemolysin

producers. *E. coli* isolates from fish, pig, broiler and human were exhibited high haemolysin titre value with the geometric mean of 6.4 against pig and human erythrocytes. The haemolysin titre of fish, pig and human isolates against goat erythrocytes was also 6.4. The geometric mean value of haemolysin titre goat isolates against the pig, human and goat erythrocytes were 5.27, 5.27 and 9.18, respectively (Table 3).

Antibiotic resistance pattern: The drug

resistance pattern of all *E. coli* isolates was studied against 20 antibiotics. *E. coli* isolates from the goat were resistant to a maximum number of antibiotics followed by chicken and broiler (Table 4). *E. coli* isolates from human were found to be resistant to cefotaxime, ceftriaxone and chloramphenicol and only intermediate sensitive to cotrimoxazole and tetracycline.

Production of Extended-spectrum beta-lactamase enzyme (ESBL): *E. coli* isolates from goat, broiler, fish and human were resistant to cefotaxime (CTX). The isolates of chicken and pig were found to be intermediate sensitive and sensitive, respectively (Table 5). Similarly,

isolates of pig and chicken were sensitive to cefotaxime and clavulinic acid (CEC) combination. On the other hand, fish isolates were resistant to cefotaxime and clavulinic acid combination. One isolate each from both goat and broiler also showed resistant activity against CEC. Among the tested isolates, two isolates of fish, one isolate from goat and broiler were found to be ESBL procedure.

DISCUSSION

E. coli is one of the most important zoonotic enteric pathogens and the most widely accepted indicator of faecal contamination in food and water. Virulence pattern of *E. coli* is often

Table 3. The geometric mean values of haemagglutination and haemolysin titre of the *E. coli* isolates against different erythrocytes

Source of <i>E. coli</i>	No. of isolates	Haemagglutination titre against erythrocytes of					Haemolysin titre against erythrocytes of				
		Fish	Broiler	Pig	Human	Goat	Fish	Broiler	Pig	Human	Goat
Fish	2	1.41	1.0	1.0	1.0	1.0	1.41	1.41	6.4	6.4	6.4
Pig	3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	6.4	6.4	6.4
Human	4	1.41	1.0	1.0	1.41	1.0	1.41	1.0	6.4	1.0	6.4
Chicken	11	1.06	1.13	1.06	1.06	1.13	1.20	1.13	6.4	1.65	11.67
Goat	10	1.31	2.0	1.0	1.0	2.0	1.31	1.14	5.27	5.27	9.18
Broiler	12	1.16	1.36	1.08	1.36	1.36	1.16	1.71	6.4	6.4	2.25

Table 4. The resistance pattern of *E. coli* isolates from different sources towards common antibiotics

Sources	Number of isolates	Antibiotics
Broiler	12	Ceftriaxone, Cefazolin, Cefotaxime, Clotrimazole, Nystatin, Penicillin, Cotrimoxazole*
Human	4	Cefotaxime, Cefazolin, Chloramphenicol, Ceftriaxone*, Tetracycline*
Fish	2	Ceftriaxone, Cefotaxime, Cefazolin, Nystatin, Clotrimazole, Penicillin, Cotrimoxazole*
Goat	10	Ampicillin, Cefotaxime, Meropenem, Cefepime, Ceftazidime, Cefpodoxime, Cefoperazone
Chicken	11	Cotrimoxazole, Ceftriaxone, Cefepime, Ceftazidime, Cefpodoxime, Cefoperazone, Cefotaxime*
Pig	3	Ceftriaxone, Cefazolin, Clotrimazole, Nystatin, Penicillin

*indicates "intermediate sensitive" bacteria

Table 5. β -Lactamase producing ability of *E. coli* isolates from various sources

Source of <i>E. coli</i>	Sensitivity pattern towards	
	Cefotaxime	Cefotaxime + Clavulanic acid
Goat1	R	S
Goat2	R	R*
Broiler1	R	R*
Broiler2	R	IS
Fish1	R	R*
Fish2	R	R*
Human1	R	IS
Human2	R	IS
Chicken1	IS	S
Chicken2	IS	S
Pig1	S	S
Pig2	S	S

*ESBL producing isolates; S - sensitive ;
IS - intermediate sensitive; R - resistant

multifactorial and, therefore, studies on virulence factors have very important practical implications. In the present investigation, *E. coli* were successfully isolated, identified and evaluated for their virulent attributes and antibiotic resistance pattern.

The congo red binding is often correlated with the pathogenicity of the organism and possibly used as a phenotypic marker to distinguish invasive and non-invasive *E. coli* (Panigrahy and Yushen, 1990; Sharma *et al.*, 2007). Berkhoff and Vinal (1986) found that about half of the environmental and cloacal isolated *E. coli* was congo red positive, while Panigrahy and Yushen (1990) found that 61.9% of the *E. coli* were congo red positive. Similarly, about 45 - 47.42% of the entero-invasive *E. coli* were able to bind congo red (Sharma *et al.*, 2007). In this study, out of 42 isolates, maximum congo red positive were from chicken (72%) and human (50%) followed by broiler (40%) and goat (40%).

In this study, all the isolates irrespective of the source of isolation were moderate to strong

biofilm producers. Earlier several researchers have demonstrated *in vitro* biofilm-forming ability of *E. coli* isolates from various sources (Dewanti and Wong, 1995; Pratt and Kolter, 1998; Sheikh *et al.*, 2001). However, *in vitro* biofilm-forming ability varies greatly among the *E. coli* isolates (Reisner *et al.*, 2006). Sleytr *et al.* (1993) documented the prevalence of biofilm production among 17% faecal *E. coli*, 43% for cystitis, 40% for pyelonephritis and 42% for bacteraemic *E. coli*. Shetty *et al.* (2014) reported 20% of the intestinal commensal *E. coli* to be biofilm formers.

Proteases play a crucial role in virulence which helps in the survival and immune evasion of the pathogen (Iqbal *et al.*, 2013). Several researchers have demonstrated the proteolytic activity of *E. coli* (Haddadi *et al.*, 2005; Budiarti and Mubarik, 2007; Abed *et al.*, 2016). In this study, we have also observed high proteolytic activity of *E. coli* isolates irrespective of the source of isolation. Earlier researchers have demonstrated high hemolysin producing ability of *E. coli* from various sources (Johnson, 1991; Mandal *et al.*, 2001; Raksha *et al.*, 2003; Sharma *et al.*, 2007; Fakruddin *et al.*, 2013; Shetty *et al.*, 2014). Raksha *et al.* (2003) reported a high percentage (41%) of haemolytic *E. coli* from urinary samples. Likewise, Shetty *et al.* (2014) reported 28.66% and 15% of extra-intestinal and intestinal commensal *E. coli* isolates to be hemolysin producer, respectively. Haemolytic activity is often associated with pathogenicity of *E. coli* (Johnson, 1991). *E. coli* strains mostly produce α -haemolysin (Shetty *et al.*, 2014; Bhugubalda *et al.*, 2016), which possibly contributes to the virulence since it conferred virulence to non-pathogenic *E. coli* strains (Elliott *et al.*, 1998). There is an existing correlation between the hemagglutinating ability and adhesiveness of several enteric pathogens (Qadri *et al.*, 1994). Further, the receptors on the erythrocytes vary from species to species and the haemagglutination activity of *E. coli* isolates could indicate a clue as to the nature of the receptors for these pathogens in the intestinal mucosa (Hagberg *et al.*, 1981; Qadri *et al.*,

1994). All the isolates were haemolysin producer with very strong haemolysin titre, which may pose a potential threat to these species.

Antibiotic resistance among the *E. coli* is rapidly increasing due to indiscriminate use of antibiotics (Bhrugubalda *et al.*, 2016). Earlier, the multidrug-resistance pattern of *E. coli* strains against ampicillin, ciprofloxacin, gentamycin, sulfamethoxazole, and tetracycline has been reported (Mohammadi-Sardo *et al.*, 2017). Besides, studies have also indicated a significant correlation between multidrug resistance pattern, biofilm formation, and pathogenesis of *E. coli* (Ponnusamy *et al.*, 2012; Poursina *et al.*, 2018). Karigoudar *et al.* (2019) found that 90.6, 71.9, 65.6, 59.3, 56.3, 56.3, 53.1 and 50.0% of biofilm-producing *E. coli* were resistant towards erythromycin, amikacin, cotrimoxazole, ampicillin, meropenem, chloramphenicol, tobramycin, and gentamicin, respectively. In this study, *E. coli* isolates were found to be resistant to at least 5 tested antibiotics except for isolates from human stool samples. The high antibiotic resistance pattern was recorded from the isolates from fish, goat and broiler. Although the exact source of such antibiotics resistant strains in the GI tract of these animals needs thorough studies, their occurrence as normal autochthonous gut microbiota of these animals could be harmful to the host especially under stressful conditions

Extended-spectrum β -lactamases (ESBLs) are enzymes that mediate resistance to extended-

spectrum cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime, and the monobactam aztreonam (Tenover *et al.*, 1999). While some researchers observed a low rate of ESBL production by *E. coli* (Bhrugubalda *et al.*, 2016), others reported a high rate of ESBL production by *E. coli* (Sharma *et al.*, 2007). In this study, *E. coli* isolates from goat, pig and chicken were found to be ESBL producers. The findings of the present study agree with the views that the virulence of an organism may not be accurately predicted based on its measurable virulence factor phenotype but the presence of multiple virulence factors does increase the virulence of organisms (Bhrugubalda *et al.*, 2016).

Therefore the present investigation showed that the normal gastrointestinal *E. coli* possess several virulent traits like biofilm-producing, congo red binding, proteolytic and haemolytic, which could be a predisposing factor for the onset of diseases in animals. Nonetheless, the ESBL positive along with the multidrug resistance pattern is a major concern since the possibility of horizontal transfer and possible health implications in the host as well as to human can't be ruled out.

Conflict of interest: The authors declare that they have no competing interests.

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