Prevalence and Characterization of *Escherichia coli* from Poultry Meat in Bhubaneswar

Iswar Anand Senapati[1*], Rajashree Mishra[1], Akshaya Kumar Kundu[2],
Bidyut Prava Mishra[3] and Prasana Kumar Rath[4]

[1]*Department of Veterinary Microbiology,* [2]*Department of Veterinary Physiology,* [3]*Department of Livestock Products Technology,* [4]*Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar, India*

*Corresponding author

A B S T R A C T

E. coli are normal inhabitants of the gastrointestinal tract of animals, birds and humans of which only some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. In the present study, a total of 320 raw poultry meat samples were collected from retail poultry meat market of Bhubaneswar, Odisha, India. A total of 224 *E. coli* isolates were obtained. The triturated samples were first inoculated on MLA and then on EMB agar, the colonies with greenish metallic sheen were considered as *E. coli* which was further confirmed by biochemical tests. The overall prevalence of *E. coli* observed during the present study was 70 per cent in the samples. *In vitro* antibiotic resistance patterns against 18 antibiotics revealed highest resistance to Oxytetracycline and highest sensitivity was exhibited to Tobramycin by all types of isolates. All the 224 *E. coli* isolates were subjected to biofilm production by Microtiter Plate Method and Congo Red Agar method. 68.75% of *E. coli* isolates were found to be positive for biofilm formation by both MTP method and CRA method. By MTP method 12.5%, 79.17% and 8.33% isolates are high, moderate and weakly positive respectively. By CRA method, 20.83%, 50% and 29.17% isolates are high, moderate and weakly positive respectively.

Introduction

Poultry meat is a highly digestible, tasty and low-calorie food, often recommended by nutritionists over other meats. Poultry meat is often contaminated with *Escherichia coli* at the time of slaughtering. *E coli* are normal inhabitants of the gastrointestinal tract of animals, birds and humans of which some strains have become highly adapted to cause

diarrhoea and a range of extra-intestinal diseases. The organism continues to provide new challenges to food safety or public health. The work has been conducted for the prevalence and characterization of *E. coli* from broiler birds' meat in and around Bhubaneswar, Odisha. They were screened for biofilm formation and antimicrobial susceptibility.

Materials and Methods

The present study was undertaken with a view to isolate *E. coli* from different samples of poultry meat sold in retail markets of Bhubaneswar and to study their properties in relation to biochemical characters, *in vitro* antimicrobial drug resistance pattern and biofilm forming capacity. The recovered isolates were confirmed on the basis of their morphological, cultural and biochemical characteristics. Altogether 320 samples comprising of breast muscle and leg muscle (160 samples each) were collected from randomly selected ten different poultry meat shops in Bhubaneswar, under aseptic measures. The samples were collected in sterile plastic containers and transported to the laboratory in an ice box as soon as possible. All samples were immediately marked post collection. Each sample was thoroughly triturated in a sterile mortar and pestle before inoculating on media for isolation. The samples thus collected were subjected to bacteriological examination. Isolation of *E. coli* was attempted from different samples of meat according to Edwards and Ewing (1972), Cruickshank *et al.,* (1975) and Quinn *et al.,* (1994) by using MacConkey lactose agar (MLA) and Eosin Methylene Blue (EMB) agar as selective media.

After receiving at laboratory the samples were unpacked aseptically. A small piece was cut out of each sample and ground using mortar and pestle with one time addition of few milliliters of normal saline. Then a 1ml of meat paste was inoculated with BHI broth. After an incubation of 18 hours the culture was considered for Gram Stain. Those cultures which revealed the presence of Gram Negative bacilli were passed on for further tests. Each sample having Gram Negative rods were inoculated on MLA and incubated at 37°C for 24 hours. The pink colonies were streaked on EMB and incubated at 37°C for 24 hours. Dark colonies with greenish nucleated metallic sheen after 24 hours of inoculation at 37°C were considered to be of typical *E. coli*. They were transferred to nutrient agar (NA) slants so as to sustain them for further identification and characterization. The identification of the isolates was done according to the methods described by Sojka (1965), Edwards and Ewings (1972), Cruickshank *et al.,* (1975) and Kreig and Holt (1984). The isolates were studied for their biochemical characters *viz.,* IMViC (Indole, Methyl Red, Voges-Proskauer and Citrate) reactions.

The antibiotic susceptibility tests were performed as per method described by Bauer *et al.,* (1966) to find out the antibiotic resistance pattern of all *E. coli* isolates. *In vitro* antibiotic sensitivity test of the isolates was conducted by paper disc diffusion method using the disc supplied by HiMedia Laboratories Pvt. Ltd., Mumbai, India. Isolates were subjected to antimicrobial sensitivity test against 18 antibiotics such as Amikacin (AK30), AmoxyClav (AMC30), Ampicillin/Cloxacillin (AX10), Cefepime (CPM30), Ceftriaxone (CTR30), Cephotaxime (CTX30), Chloramphenicol (C30), Ciprofloxacin (CIP5), Co-Trimoxazole (COT25), Doxyxycline (DO10), Gentamicin (GEN10), Imipenem (IPM10), Nalidixic acid (NA30), Nitrofurantoin (NIT200), Norfloxacin (NX10), Oxytetracycline (O30), Streptomycin (S10) and Tobramycin (TOB10).

E. coli isolates were grown in Brain Heart Infusion (BHI) broth (HiMedia) for 12-18 hours. The grown cultures were swabbed on Mueller Hinton agar plate (HiMedia) using sterile cotton swabs and left for at least 5 minutes as pre-diffusion time. Then using an ethanol dipped and flamed forceps different antibiotic discs were placed on the agar

surface at about two centimeters apart. The discs were slightly pressed with the forceps to make complete contact with the medium. The plates were incubated at 37°C for 18-24 hours. After the incubation period, the diameter of inhibition zones were measured and compared with interpretative chart provided by the manufacturer and zones were graded as sensitive, intermediate and resistant.

Biofilm formation assay was performed by tissue culture plate method according to the protocol described by O'Toole G.A. (2011). The cultures of *Escherichia coli* were grown for 24 hours at 37°C in Tryptone Soya Broth (TSB). Cultures were added in the wells of the microtiter plate. The microtiter plate was incubated for 8 hours at 37°C. After incubation the cells were dumped out by turning the plate over and shaking out the liquid. The plate was gently submerged in a small tub of water and the water was shaken out. The process was repeated for a second time. This step helps remove unattached cells and media components that can be stained in the next step and significantly lowers the background staining. 125 µl of a 0.1% solution of crystal violet in water was added to each well of the microtiter plate. The microtiter plate was incubated at room temperature for 15 minutes. The plate was rinsed 4 times with water by submerging in a tub of water followed by shaking out and blotting vigorously on a stack of paper towel to rid the plate of all excess cells and dye. The microtiter plate was turned upside down and left for drying. For qualitative assay the wells were photographed after drying. Freeman *et al.,* (1989) method was followed with slight modification. The Medium was composed of Tryptone soya agar (HiMedia, Mumbai) 40g/l, sucrose 50g/l and Congo red 0.8g/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents, and was then added when the agar had cooled to 55°C.

Plates of the medium were inoculated and incubated aerobically for 24 hours at 37°C. A positive result was indicated by black colonies with dry crystalline consistency. Non-slime producers usually remained pink, though occasional darkening at the centre of the colonies was observed and this gave a bulls eye appearance. An indeterminate result was indicated by a darkening of the colonies but with the absence of a dry crystalline colonial morphology. Isolates presenting two tones of black, bright black (BB) and dry opaque black (OB), were classified as negative.

Results and Discussion

Isolation and Identification of *E. coli* isolates

During present study, an attempt was made to isolate *E. coli* from the raw poultry meat samples from retail meat market of Bhubaneswar. 224 (70.00%) *E. coli* isolates were obtained from 320 raw poultry meat samples. All the isolates revealed characteristic features of *E. coli*, which were Gram negative bacilli, by producing characteristic pink colonies on MLA and colonies with greenish metallic sheen on eosin methylene blue agar (EMB). On preliminary biochemical characterization they revealed characteristic IMViC pattern that is + + - -. Almost similar result was found by Nzouankeu *et al.,* (2010), who collected 150 poultry meat samples from eight retail markets in Yaounde. However, lower isolation rate was reported by Dutta *et al.,* (2011) who also recovered *E. coli* isolates from chickens. The report of isolation of *E. coli* by various workers; Silva *et al.,* (2011) isolated *Escherichia coli* from chicken livers from two slaughter houses were genotypically characterized in 62 samples. Thirty samples were macroscopically unchanged and 32 demonstrated alterations that led to the disposal of carcass for sanitary inspection.

Thirty *Escherichia coli* strains from 21 unchanged and 9 from carcasses that were rejected were isolated through the classical method. Zhao *et al.,* (2012) recovered *Escherichia coli* from the National Antimicrobial Resistance Monitoring System retail meat program and examined for antimicrobial susceptibility. They screened 11,921 retail meat samples from four U.S. states collected during 2002–2008 which consist of 2,988 chicken breasts. They found that 83.5 per cent chickens were contaminated with the organism.

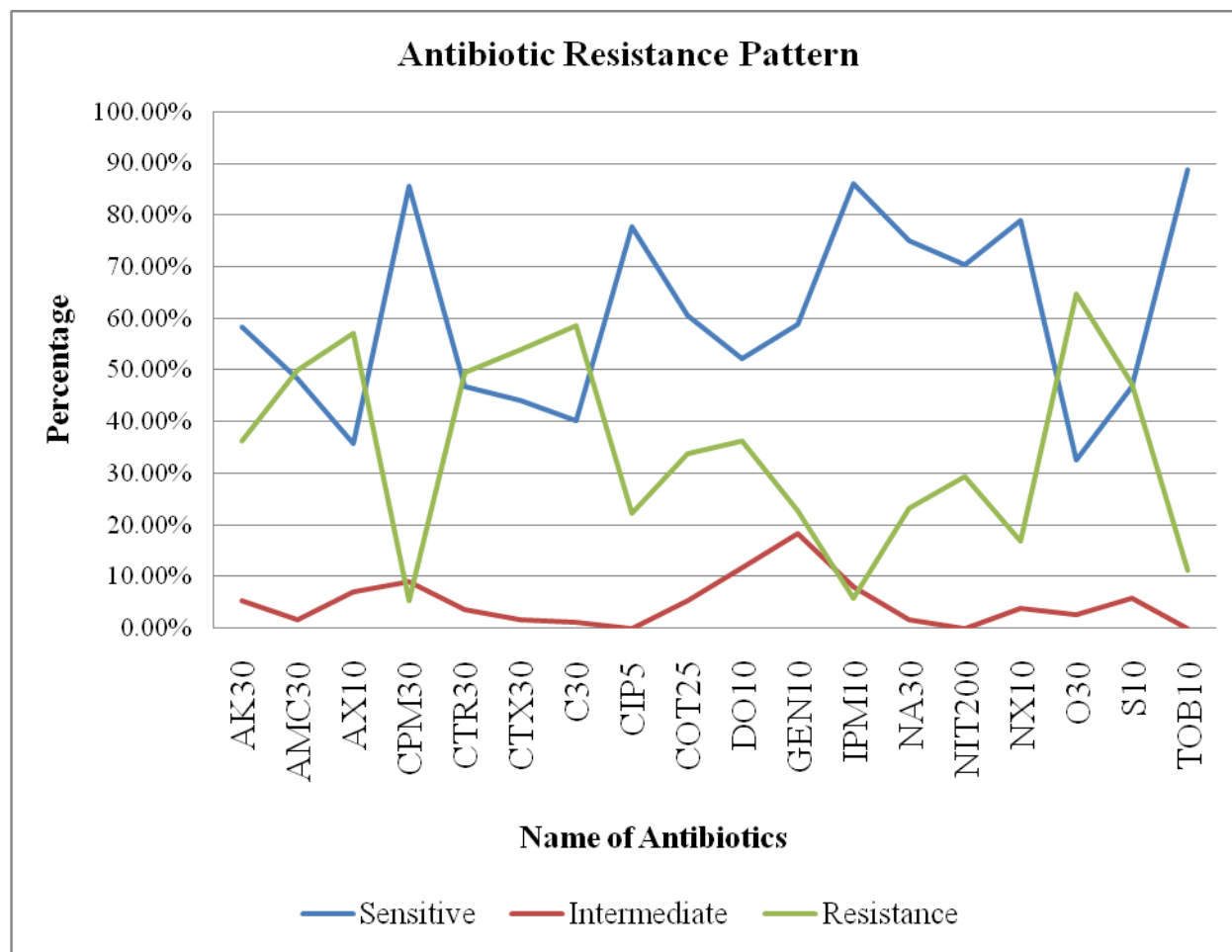## Antimicrobial drug resistance among the *E. coli* isolates

Among 224 *E. coli* isolates the highest resistance was observed against the Oxytetracycline (64.73%) followed by Chloramphenicol (58.48%), Ampicillin/ Cloxacillin (57.14%), Cephotaxime (54.02%), Amoxycillin + Clavulanic acid (50.00%), Ceftriaxone (49.55%), Streptomycin (47.32%), Doxyxycline (36.16%), Amikacin (36.16%), Co-Trimoxazole (33.93%), Nitrofurantoin (29.46%), Nalidixic acid (23.21%), Gentamicin (22.78%), Ciprofloxacin (22.32%), Norfloxacin (16.96%), Tobramycin (11.16%), Imipenem (5.80%) and least resistance was observed against Cefepime (5.36%). Highest sensitivity was exhibited to Tobramycin (88.84%) followed by Imipenem (86.16%), Cefepime (85.71%), Norfloxacin (79.02%), Ciprofloxacin (77.68%), Nalidixic acid (75.00%), Nitrofurantoin (70.54%), Co-Trimoxazole (60.71%), Gentamicin (58.93%), Amikacin (58.48%), Doxyxycline (52.23%), Streptomycin (46.88%), Amoxycillin + Clavulanic acid (48.21%), Ceftriaxone (46.88%), Cephotaxime (44.20%), Chloramphenicol (40.18%), Ampicillin/ Cloxacillin (35.71%) and Oxytetracycline (32.59%). This is being depicted in Table 1 and Figure 1.

In the present study highest per cent of isolates (64.73%) were resistant to oxytetracycline. While lower resistance was reported by Singh *et al.,* (1992) and Bogaard *et al.,* (2001). Moderately higher resistance of 58.48% has been noticed against Chloramphenicol. Similar findings have also been reported by Cid *et al.,* (1996). Higher resistance was reported by. While lower resistance was reported by Sharada *et al.,* (2010), Dash *et al.,* (2012), Zhao *et al.,* (2012), Pavlickova *et al.,* (2017) and Somda *et al.,* (2018). Moderate per cent (57.14%) of isolates were found resistant to Ampicillin/Cloxacillin. Similar findings have also been reported by Singh *et al.,* (1992) and Somda *et al.,* (2018). The results are in contrast to the higher resistance as reported by Cid *et al.,* (1996), Dash *et al.,* (2012) and Suzuki *et al.,* (2019). Lower resistance has been reported by Aksoy *et al.,* (2007), Zhao *et al.,* (2012) and Pavlickova *et al.,* (2017). In the present study moderate per cent (54.02%) of isolates were found resistant to Cephotaxime. Lower resistance has been reported by Aksoy *et al.,* (2007), Dash *et al.,* (2012), Pavlickova *et al.,* (2017), Somda *et al.,* (2018) and Bantawa *et al.,* (2019). 50.00% isolates were found resistant to Amoxycillin + Clavulanic acid. The result is in accordance to moderate resistance as reported by Somda *et al.,* (2018). Bantawa *et al.,* (2019) reported 100% resistance to amoxycillin. In contrast to the findings, lower resistance was reported by Aksoy *et al.,* (2007), Zhao *et al.,* (2012) and Pavlickova *et al.,* (2017). In the study moderate per cent (49.55%) of isolates were found resistant to Ceftriaxone. Similar findings have also been reported by Singh *et al.,* (1992) and Somda *et al.,* (2018). The results are in contrast to the higher resistance as reported by Cid *et al.,* (1996), Dash *et al.,* (2012) and Suzuki *et al.,* (2019).

Table.1 *In vitro* antimicrobial drug resistance pattern of *E. coli* isolates

Sl. No.	Antimicrobial agents	Sensitive	Intermediate	Resistance
1	Amikacin (AK)	131 (58.48%)	12 (5.36%)	81 (36.16%)
2	Amoxycillin + Clavulanic acid (AMC)	108 (48.21%)	4 (1.79%)	112 (50.00%)
3	Ampicillin/Cloxacillin (AX)	80 (35.71%)	16 (7.14%)	128 (57.14%)
4	Cefepime (CPM)	192 (85.71%)	20 (8.93%)	12 (5.36%)
5	Ceftriaxone (CTR)	105 (46.88%)	8 (3.57%)	111 (49.55%)
6	Cephotaxime (CTX)	99 (44.20%)	4 (1.78%)	121 (54.02%)
7	Chloramphenicol (C)	90 (40.18%)	3 (1.34%)	131 (58.48%)
8	Ciprofloxacin (CIP)	174 (77.68%)	0 (0.00%)	50 (22.32%)
9	Co-Trimoxazole (COT)	136 (60.71%)	12 (5.36%)	76 (33.93%)
10	Doxyxycline Hydrochloride (DO)	117 (52.23%)	26 (11.61%)	81 (36.16%)
11	Gentamicin (GEN)	132 (58.93%)	41 (18.30%)	51 (22.78%)
12	Imipenem (IPM)	193 (86.16%)	18 (8.04%)	13 (5.80%)
13	Nalidixic acid (NA)	168 (75.00%)	4 (1.78%)	52 (23.21%)
14	Nitrofurantoin (NIT)	158 (70.54%)	0 (0.00%)	66 (29.46%)
15	Norfloxacin (NX)	177 (79.02%)	9 (4.02%)	38 (16.96%)
16	Oxytetracycline (O)	73 (32.59%)	6 (2.68%)	145 (64.73%)
17	Streptomycin (S)	105 (46.88%)	13 (5.80%)	106 (47.32%)
18	Tobramycin (TOB)	199 (88.84%)	0 (0.00%)	25 (11.16%)

Figure.1 *In vitro* antimicrobial drug resistance pattern of *E. coli* isolates

Lower resistance has been reported by Zhao *et al.,* (2012) and Somda *et al.,* (2018). 47.32% of isolates had shown resistance to Streptomycin in this study. Higher resistance was reported by Cid *et al.,* (1996) and Schroeder *et al.,* (2002). Dash *et al.,* (2012) observed 100% resistance towards Streptomycin. Lower resistance was observed by Aksoy *et al.,* (2007) and Zhao *et al.,* (2012). Doxycycline was found to have resistance of 36.16% and a sensitivity of 52.23% in the present study. In contrast to those, Pavlickova *et al.,* (2017) found less resistant and more sensitive isolates. Highest number (88.84%) of isolates in this study are sensitive to Tobramycin. It is in agreement with Cid *et al.,* (1996). In our study Imipenem

holds the second position in terms of number of isolates being sensitive to it (86.16%). It is in accordance with Pavlickova *et al.,* (2017). Somda *et al.,* (2018) reported 100% sensitivity towards Imipenem. In present study 85.71% of isolates were found to be sensitive to Cefepime which is in agreement with Pavlickova *et al.,* (2017). Norfloxacin received sensitivity from 79.02% isolates. It is in accordance with Sharada *et al.,* (2010). In contrast quite lower sensitivity was obtained by Alimehr *et al.,* (1999). For Ciprofloxacin, 77.68% sensitivity of isolates was observed in this study which is in accordance with Sharada *et al.,* (2010), Dash *et al.,* (2012) and Pavlickova *et al.,* (2017). Higher sensitivity was observed by Zhao *et al.,* (2012) and

Somda *et al.,* (2018). In this study 75% isolates are sensitive towards Nalidixic acid. It is in accordance with Bantawa *et al.,* (2019). Higher values are obtained by Cid *et al.,* (1996), Zhao *et al.,* (2012) and Somda *et al.,* (2018). 70.54% isolates are sensitive to Nitrofurantoin in this study. Higher sensitivity was observed by Karlowsky *et al.,* (2003) and Zykov *et al.,* (2015). Co-trimoxazole had sensitivity of 60.71% isolates. It is in accordance with Cid *et al.,* (1996) and Somda *et al.,* (2018). Higher sensitivity was observed by Schroeder *et al.,* (2002), Aksoy *et al.,* (2007) and Pavlickova *et al.,* (2017).

Lower sensitivity was reported by Sharada *et al.,* (2010). Gentamicin was sensitive to 58.93% isolates in this study. Higher sensitivity was projected by Singh *et al.,* (1992), Cid *et al.,* (1996), Schroeder *et al.,* (2002), Aksoy *et al.,* (2007), Zhao *et al.,* (2012) and Somda *et al.,* (2018). In contrast to these, lower sensitivity was observed by Dash *et al.,* (2012) and Pavlickova *et al.,* (2017). In this study Amikacin got sensitivity from 58.48% isolates. It is in accordance with Dash *et al.,* (2012). Higher sensitivity was reported by Cid *et al.,* (1996).

Biofilm formation assay

There is growing appreciation that formation of bacterial surface communities is a process that contributes to pathogenicity of microorganisms (Parsek *et al.,* 2004). It has become a common working hypothesis that the persistence of bacterial biofilms in the human body is a major cause of recurrent or chronic infections (Hall-Stoodley *et al.,* 2005). In addition, formation of bacterial associations within bladder epithelium is characteristic of experimental urinary tract infections (UTI) (Kau *et al.,* 2005). Many such reports are being published, from all over the world, revealing biofilm forming pathogenic *E.* coli an emerging threat to

animal as well as public health. Since most of the presently available antibiotics are ineffective against such exopolysaccharide, it could lead to a crisis in public health in near future. That's why biofilm formation assay was being conducted under the present study. All the 224 *E. coli* isolates were subjected to biofilm production by Microtiter Plate Method and Congo Red Agar method.

In Microtiter plate method, 154 out of 224 isolates (68.75%) were found to be positive for biofilm formation. Among those, 7.14% isolates are highly positive, 70.78% isolates are moderately positive and 27.08% are weakly positive. Also, in Congo Red Agar method, 68.75% isolates produced biofilm. 23.38% isolates are highly positive, 37.66% isolates are moderate positive and 38.96 % are weakly positive.

Summary and conclusions are as follows:

The majority of market poultry meat samples were found to be contaminated or carried *E. coli* infection.

Around 70% of meat samples were carrying detectable level of *E. coli* which is quite a high rate of prevalence, which itself carries a public health concern. Antibiotic sensitivity testing of *E. coli* isolates revealed highest resistance of 64.73% to Oxytetracycline followed by Chloramphenicol (58.48%), Ampicillin/C loxacillin (57.14%) and so on. Highest sensitivity was recorded with Tobramycin (88.84%) followed by Imipenem (86.16%), Cefepime (85.71%), Norfloxacin (79.02%) and so on. This much of resistance indicates the indiscriminate and widespread use of these antimicrobials as feed additives or for prophylactic purpose in poultry industry, emphasizing strict and urgent need of restrictions in order to make the use of antibiotics judicious.

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