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Research Article

A Search for Identifying Aerobic Bacteria by Culture and Multiplex PCR in Market Eggs Causing Gastroenteritis and Enteric Fever in Bangladesh

Jannatul Fardows[1] and SM Shamsuzzaman[2]*

[1]Department of Microbiology, Dhaka Medical College, Bangladesh
[2]Department of Microbiology, Dhaka Medical College, Bangladesh

*Corresponding author

SM Shamsuzzaman, Professor, Department of Microbiology, Dhaka Medical College, Dhaka 1000, Bangladesh, Tel: 880-29665518; Fax: 880-1819289739; Email: smszaman@yahoo.com

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Abstract

To observe the chance of possible transmission of pathogenic bacteria from market egg to the community, potential pathogenic aerobic bacteria were detected from market eggs by culture and multiplex PCR. Egg shells and egg contents of 150 eggs collected from different markets of Dhaka city were tested. Total 145 (96.67%) egg shells yielded growth of bacteria, 23 (15.86%) of them were ESBL producers. *Esch. coli* was the most common (26.67%) bacteria and 7 (4.67%) were *Salmonella spp.* Other bacteria were *Klebsiella pneumoniae* (6.67%), *Proteus vulgaris* (3.33%), *Proteus mirabilis* (2%), *Providencia rettgeri* (15.33%), *Providencia alkalifaciens* (1.33%), *Acinetobacter baumanii* (8.67%), *Citrobacter freundii (10%), Enterobacter aerogenes (6.67%) Klebsiella oxytoca* (4.67%) and *Pseudomonas aeruginosa* (6.67%). By PCR, 15 (10%) *Salmonella spp.* was identified from egg shells and the most common serotype was *Salmonella* Enteritidis (53.33%). No bacteria were detected from egg contents. Most of the bacteria were sensitive to imipenem and colistin. All *Salmonella* serotypes were sensitive to chloramphenicol, imipenem, gentamicin, ciprofloxacin and ceftriaxone. In conclusion, it can be said that market eggs may be an important source of infection of many gram negative bacteria including *Salmonella* to the community.

INTRODUCTION

Eggs and egg products are nutritive food items and a vital constituent of human food in the world [1]. They are rich in protein, phosphorous, selenium, choline, riboflavin, vitamin B_{12}, folic acid, zinc, pantothenic acid and vitamin A, D, E and K [2]. Inaccurately treated eggs can cause food-borne illness [3]. The intestinal tract of hen is the reservoir of *Salmonella spp.* as well as other microorganisms which infects human [4]. The absence of standard structures and drainage system in the market and relatively high humidity could have contributed to the high microbial growth [5]. Most retailers do not store eggs in refrigerators, thus the eggs are exposed to weather conditions, resulting in their contamination [6]. Microbial contamination of egg has important outcome to the poultry industry and illness from contaminated egg is a serious public health problem around the world [1]. In spite of the antibacterial factors, it can be infected with different bacteria such as *Salmonella spp., Esch coli, Listeria monocytogens, Campylobacter jejuni, Proteus spp.* and *Klebsiella spp* [7]. The shell acquires infection from all surface with which it makes contact. The extent of infection is directly related to the cleanliness of these surfaces, and storage under very humid conditions [8].

It is estimated that in the U.S. *Salmonella* transmission through contaminated egg shell or egg products results in 48 million cases of salmonellosis and costs $ 365 million annually [9]. Enteric fever and gastroenteritis is a common disease in Bangladesh. Eggs are common food item in every house hold and it may be assumed that eggs may be one of the sources of such infections in Bangladeshi community. Different *Salmonella* species identified by culture from egg shells have been reported in Bangladesh [10]. But other potential pathogenic bacteria have not been reported yet and no such study has been done on egg contents from market eggs in the country. The present study is carried out to isolate the pathogenic aerobic bacteria including *Salmonella* from egg shell and egg contents of hen by culture and multiplex PCR and to see their antimicrobial susceptibility pattern.

MATERIALS AND METHODS

This cross sectional study was carried out on egg shells and egg contents of 150 eggs collected from different markets of Dhaka City of Bangladesh. It was done in the department of Microbiology, Dhaka Medical College (DMC), Dhaka, Bangladesh from July, 2012 to June, 2013. Undamaged and clean eggs without fecal contamination and cracks were included in this study.

Sample processing with pre-enrichment and enrichment

For each egg, one sterile swab stick was made wet by TSB. One sterile swab stick was made wet by peptone water broth and shell swab was taken from the entire surface of the egg and was immediately inoculated in a test tube containing 9 ml peptone water broth for pre-enrichment for *Salmonella spp.* Another shell swab was taken from the entire surface of the egg and was immediately inoculated in a test tube containing 9 ml TSB for enrichment for aerobic bacteria other than *Salmonella spp* [11]. After collection of shell swabs, same egg was taken for egg contents (egg yolk and white part). Egg surface was sterilized by immersion in 70% alcohol for 2 min in a sterile dish, air dried in a sterile paper for 10 min, then cracked by a sterile spoon. Then carefully egg contents were taken in other sterile dish and mixed thoroughly by sterile mixer. Then, one ml of the mixed egg contents were immediately inoculated in a test tube containing 9 ml TSB for enrichment for aerobic bacteria and one ml egg contents were inoculated in a test tube containing 9 ml peptone water broth for pre-enrichment for *Salmonella* by sterile disposable syringe [12].

Isolation of organisms

After processing, the enriched (TSB) and pre-enriched media (PWB) were incubated at 37°C for 24 hours. Then one or two loopful inoculum was streaked on MacConkey's agar media and blood agar media from TSB and again incubated at 37°C for 24 hours. Subculture was done by taking one ml peptone water broth culture to a test tube containing fresh TSB and incubated for 24 hours at 37°C for enrichment for *Salmonella spp*. Then one or two loopful inoculum was streaked on MacConkey's agar media, XLD agar media and *Salmonella-Shigella* agar media from TSB and incubated at 37°C for 24 hours. All the plates were examined after 24 hours for visible colony of bacteria [11,12].

Identification of organisms

All the isolated organisms were identified by their colony morphology, staining characters and further confirmed by relevant biochemical tests including oxidase test, reactions in Triple Sugar Iron (TSI) agar media, Simmon's citrate agar media, motility indole urea (MIU) media and sugar fermentation test. *Esch. coli* was identified by observing pink colony on MacConkey's agar media with acid slant and butt in TSI media, motile, indole positive and urease negative reaction in MIU media with oxidase and citrate negative. *Salmonella* species were identified as pale colony in MacConkey's agar media with acid butt and alkaline slant with or without H_2S and gas production in TSI media, indole, urease and oxidase negative and sugar (lactose & sucrose) fermentation test negative [10].

Antibiotic susceptibility test

Using Kirby-Bauer modified disc-diffusion technique, antibiotic susceptibility test was performed and as described by Clinical and Laboratory standards Institute [13,14]. Antibiotics used were ceftazidime (30µg/disc), cetriaxone (30µg/disc), imipenem (10µg/disc), amoxiclav (amoxicillin and clavulanic acid), (20/10µg), ciprofloxacin (5µg/disc), amikacin (30µg/disc), colistin sulphate (10µg/disc), cefixime (30µg/disc), gentamicin (10µg/disc), piperacillin (30µg/disc), carbenicillin (30µg/disc), (chloramphenicol (30µg/disc), azithromycin (15µg/disc) and nalidixic acid (30µg/disc) (Oxoid Ltd. UK). Pure colonies of isolated organisms were emulsified in normal saline and turbidity was matched with 0.5 McFarland turbidity standards. Selected antibiotic discs were placed on inoculated Mueller Hinton agar media. These plates were incubated at 37°C for 24 hours. Resistant and sensitive bacteria were defined according to CLSI guidelines.

Detection of ESBL producing bacteria by double disc synergy assay

Disc containing 30µg of ceftazidime and a disc containing amoxicillin plus clavulanic acid (20µg + 10µg) were placed 15 to 20 mm apart from center to center on Muller Hinton agar media and the plate was incubated at 37°C for 24 hours. A clear extension of the edge of inhibition zone of cephalosporin disc towards amoxicillin plus clavulanic acid disc was interpreted as ESBL production [15].

Polymerase chain reaction (PCR)

1.5 ml pre-enriched peptone water broth was taken in a sterile micro-centrifuge tube, vortexed until mixing and centrifuged at 10000 g for 10 minutes, supernatant were discarded and the pellets were re-suspended in 100 µl sterile distilled water, heated at 100°C for 10 minutes in a heat block, then immediately placed on ice for 5 minutes and centrifuged at 14000 g at 4°C for 10 minutes, supernatant were taken into another micro-centrifuge tube and were used for DNA template for PCR [12]. At first *Salmonella spp.* was identified by detecting genus specific *invA* gene. Then different *Salmonella* serotypes were identified by detecting different serotypes specific genes. The following cycling parameters were used: initial denaturation at 94°C for 10 minutes, then 35 cycles each consisted of denaturation at 94°C for one minute, annealing for 60 seconds at 65°C for *Salmonella spp.* and *Salmonella* Typhimurium, 56°C for *Salmonella* Enteritidis, 55°C for *Salmonella* Typhi and *Salmonella* Paratyphi A and extension at 72°C for 30 seconds. After 35 cycles, one cycle of final extension was done at 72°C for 10 minutes. Electrophoresis was done at 100 volts for 35 minutes after loading in to 1.5% agarose gel, stained with 1% ethidium bromide, destained in distilled water for 20 min and visualized under UV transillumination. Primers with base pairs used in this study are s in shown in table 1.

Data analysis

After compiling data were analyzed using `Microsoft Office Excel 2007 program and X^2 test was used to compare the results.

Table 1: Serotypes of *Salmonella* with their genes, primers and their amplified product used in the study.

Name	Genes	Pr primers	Sequence (5'-3')	Base pair
Salmonella spp.	***invA***[16]	fliC-s	F-ATAGCCATCTTTACCAGTTCCCCC	284 bp
		fliC-as	R-GCTGCAACTGTTACAGGAATATGCC	
***Salmonella* Typhimurium**	***fliC***[16]	fliC-s	F- ATAGCCATCTTTACCAGTTCCCCC	183 bp
		fliC-as	R- GCTGCAACTGTTACAGGAATATGCC	
***Salmonella* Enteritidis**	***sefA***[17]	SEFA2	F-GCAGCGGTTACTATTGCAGC	310 bp
		SEFA4	R-TGTACAGGGACATTTAGCG	
***Salmonella* Typhi (O antigen)**	***tyv***[18]	tyv-s	F-GAGGAAGGGAAATGAAGCTTTT	615 bp
		tyv-as	R- TAGCAAACTGTCTCCCACCATAC	
***Salmonella* Paratyphi A (O antigen)**	***prt***[18]	parat-s	F-CTTGCTATGGAAGACATAACGAAC	258 bp
		parat-as	R-CGTCTCCATCAAAAGCTCCATAGA	
***Salmonella* Typhi (H antigen)**	***fliC***[18]	fliCcom-s	F-AATCAACAACAACCTGCAGCG	750 bp
		fliCd-as	R-GCATAGCCACCATCAATAACC	
***Salmonella* Paratyphi A (H antigen)**	***fliC***[18]	fliCcom-s	F-AATCAACAACAACCTGCAGCG	329 bp
		fliCa-as	R-TAGTGCTTAATGTAGCCGAAGG	

Table 2: Frequencies of microbial isolates from egg shells among market eggs (n=150) and distribution of ESBL producing bacteria by DDS test among them.

Types of isolates	n (%)	ESBL producer's n (%)
Esch. coli	40 (26.67)	9 (39.13)
Providencia rettgeri	23 (15.33)	4 (17.39)
Providencia alkalifaciens	2 (1.33)	1 (4.35)
Citrobacter freundii	15 (10.00)	-
Enterobacter aerogenes	10 (6.67)	-
Klebsiella pneumonia	10 (6.67)	2 (8.70)
Klebsiella oxytoca	7 (4.67)	-
Pseudomonas aeruginosa	10 (6.67)	3 (13.04)
Acinetobacter baumanii	13 (8.67)	-
Proteus vulgaris	5 (3.33)	3 (13.04)
Proteus mirabilis	3 (2.00)	1 (4.35)
Salmonella Typhi	1 (0.67)	-
Salmonella Paratyphi A	1 (0.67)	-
Others *Salmonella*	5 (3.33)	-
Total	145 (96.67)	23 (100)

Table 3: Identification of different *Salmonella* serotypes by PCR among the samples which were positive for *Salmonella* DNA in market egg shells (n=15).

Name of serotypes	Number of positive	Percentage
Salmonella Enteritidis	8	53.33
Salmonella Typhimurium	2	13.33
Salmonella Typhi	1	6.67
Salmonella Paratyphi A	1	6.67
Unidentified *Salmonella*	3	20.00
Total	15	100.00

RESULTS

Although no egg contents of eggs collected from market yielded growth of any bacteria, 145 (96.67%) egg shells yielded growth of different bacteria. Among the isolated aerobic bacteria *Esch. coli* was the most common organism (26.67%) and 7 (4.67%) were *Salmonella spp*. (Table 2). Tweenty three (15.86%) ESBL producing bacteria were identified from 145 gram negative bacteria.

Total 15 (10.00%) egg shells were positive for *Salmonella* by PCR and all the egg contents were negative for *Salmonella* DNA (Table 3). *Salmonella* Enteritidis was the most common *Salmonella* serotype (53.33%) detected from egg shells by PCR (Figure 1).

Of the 150 egg shells, 6 (85.71%) were positive by both culture and PCR (Table 4). Considering culture as gold standard, the sensitivity of PCR was 85.71%, specificity was 93.70%, positive predictive value was 40%, negative predictive value was 99.25% and accuracy was 93.33%. PCR detected significantly higher number of *Salmonella* than culture (p<0.001).

Most of the gram negative bacteria other than *Salmonella* were resistant to ciprofloxacin (Table 5). All *Salmonella* serotypes were sensitive to chloramphenicol, imipenem, gentamicin, ciprofloxacin and ceftriaxone (Table 6).

DISCUSSION

Microbial contamination of egg has important outcome to the poultry industry and illness from contaminated egg is a serious public health problem around the world [5]. Though all gram negative bacteria can contaminate eggs but *Salmonella* is a major food-borne pathogen worldwide and contaminates poultry products especially eggs and egg products [19]. Gastroenteritis and enteric fever are the major causes of infections transmitted by feco oral route in Bangladesh. Eggs are considered as a major source of protein and vitamins for everybody. Eggs are bought from the market and brought every kitchen irrespective of assessing whether it carries harmful bacteria or not. So the gastroenteritis and enteric fever germs may be transmitted from contaminated eggs.

Table 4: Comparison between results of culture and PCR for *Salmonella spp.* in egg shells among eggs collected from markets.

	Culture			
PCR	Positive n (%)		Negative n (%)	Total n (%)
Positive	6 (85.71)		9 (6.29)	15 (10.00)
Negative	1 (14.29)		134 (93.71)	135 (90.00)
Total	7 (100.00)		143 (100.00)	150 (100.00)

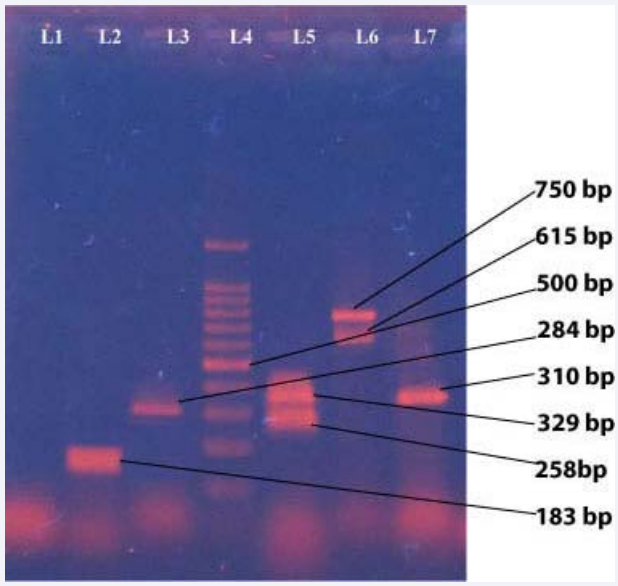

Figure 1 Photograph of amplified DNA of different serotypes of *Salmonella.* Negative control *Esch. coli* ATCC 25922 (lane 1). Amplified DNA of 183 bp for *fliC* gene of *Salmonella* Typhimurium. (lane 2), 284 bp for *invA* gene of *Salmonella spp.* (lane 3), 329 bp and 258 bp for *fliC* and *prt* gene of *Salmonella* Paratyphi A (lane 5), 750 bp and 615 bp for *fliC* and *tyv* gene of *Salmonella* Typhi respectively (lane 6) and 310 bp for *sefA* gene of *Salmonella* Enteritidis (lane 7). Hundred base pair DNA (lane 4).

In the present study, 96.67% egg shells yielded growth of pathogenic bacteria which coincide with a study where it was reported that 95% egg shells yielded growth of different bacteria from eggs collected from market [11]. These bacterial contaminations might be from cloths and hands of poultry and market workers, market retailers, use of same tray and environment of the market [20]. In the developing countries especially Bangladesh, during the market storing, inadequate refrigeration even no refrigeration can increase the percentage of different bacterial contamination on egg shell.

In the present study, among the isolated aerobic bacteria, 26.67% were *Esch. coli*. In Nigeria, relatively higher percentage of *Esch.coli* (27.5%) was observed [21]. Though *Esch. coli* is a normal flora of intestinal tract of birds and it is of low risk for human as it is also a member of normal commensal of human gut but chickens may be colonized with enterohaemorrhagic *Esch. coli* O157: H7 strain (EHEC), transmission of which in human may cause severe haemorrhagic colitis and haemolytic uremic syndrome [11]. Other diarrhoeagenic *Esch. coli* like enterotoxigenic *Esch. coli (ETEC)*, enteropathogenic *Esch.coli* (EPEC), enteroinvasive *Esch. coli* (EIEC), enteroaggregative *Esch. coli* (EagEC) and diffusely adherent *Esch. coli* may also contaminate egg shell from the farm handlers and environment and may cause watery and bloody diarrhoea. In this study, however, attempt to detect these diarrhoeagenic strains was not made.

Enteric fever is endemic in Indian subcontinent including Bangladesh [22]. It is known that *Salmonella* transmission occurs mainly by food and drink. So, egg might be an important source of *Salmonella* transmission. In addition to other gram negative bacteria, 7 (4.67%) *Salmonella spp.* was isolated by culture and 15 (10%) Salmonella DNA were detected by PCR in this study. Previous studies in Bangladesh reported 8%-12% *Salmonella* from egg. [10,23]. However, prevalence of different *Salmonella* serotype was not reported in detail in those studies.

Drug resistance is a major problem in treating the infectious diseases and drug resistance pattern among the isolated organisms have been evaluated in the present study. Among the isolated bacteria in this study, 15.86% were ESBL producers and 6.21% of them were *Esch. coli*. In contrast to the present study, no ESBL producers were observed among 72 egg shells [24]. The exact cause of absence of ESBL producing bacteria in that study is not known. In the EU, washing egg procedures are not allowed, but other processes (gaseous ozone, chloride ultraviolet radiation, dirty eggs exclusion) are applied in order to reduce the coliform load, which may contribute to the absence of ESBL producers in egg shells in those countries [25,26]. But in our

Table 5: Antimicrobial resistance pattern of isolated gram negative bacteria to different antibiotics.

Antimicrobial Drugs	*Esch. coli* (n=40)	*Providencia spp.* (n=25)	*Citrobacter freundii* (n=15)	*Klebsiella spp.* (n=17)	*Proteus spp.* (n=8)	*Enterobacter aerogenes* (n=10)	*Acinetobacter baumanii* (n=13)	*Pseudomonas aeruginosa* (n=10)
Imipenem	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (15.38)	3 (30.00)
Amikacin	14 (35.00)	15 (60.00)	10 (66.67)	10 (58.82)	6 (75.00)	5 (50.00)	13 (100.00)	8 (80.00)
Ciprofloxacin	35 (87.50)	22 (88.88)	13 (86.67)	15 (88.24)	7 (87.50)	8 (90.00)	13 (100.00)	10 (100.00)
Ceftriaxone	10 (25.00)	12 (48.00)	10 (66.67)	5 (29.41)	3 (37.50)	3 (30.00)	11 (84.62)	8 (80.00)
Ceftazidime	20 (50.00)	10 (40.00)	8 (53.33)	5 (29.41)	3 (37.50)	3 (30.00)	11 (84.62)	7 (70.00)
Cefixime	20 (50.00)	10 (40.00)	8 (53.33)	5 (29.41)	5 (62.50)	2 (20.00)	13 (100.00)	8 (80.00)
Gentamicin	10 (25.00)	15 (60.00)	10 (66.67)	12 (70.59)	5 (62.50)	7 (70.00)	13 (100.00)	7 (70.00)
Colistin	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (20.00)
Amoxyclav (amoxicillin and clavulanic acid)	25 (62.50)	20 (80.00)	12 (80.00)	10 (58.82)	6 (75.00)	6 (60.00)	13 (100.00)	8 (80.00)
PiPiperacillin	–	–	–	–	–	–	–	3 (30.00)
CaCarbenicillin	–	–	–	–	–	–	–	10 (100.00)

Table 6: Antimicrobial resistance pattern of different serotypes of *Salmonella.*

A Antibiotics	*S.* Enteritidis n (%)	*S.*Typhimurium n (%)	*S.* Typhi n (%)	*S.* Paratyphi A n (%)
Chloramphenicol	0(0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Nalidixic acid	7 (83.33)	2 (100.00)	0 (0.00)	0 (0.00)
Imipenem	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Amikacin	4 (50.00)	2 (100.00)	1 (100.00)	1 (100.00)
Ciprofloxacin	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Gentamicin	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Ceftriaxone	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Azithromycin	6 (33.33)	0 (0.00)	0 (0.00)	0 (0.00)
Amoxyclav (amoxicillin and clavulanic acid)	7 (83.33)	2 (100.00)	1 (100.00)	0 (0.00)

country, unhygienic practice of market retailers and unethical practice of antibiotics may increase the egg shell contamination and similarly increase the ESBL carrying bacteria in the community. However, Smet *et al.* observed 45% ESBL producers in cloacal sample of broilers in their study where all isolates were *Esch. Coli* [26].

In the present study, most of the identified *Salmonella* species were gastroenteritis producing *Salmonella (53.33% Salmonella* Enteritidis and 13.33% *Salmonella* Typhimurium). Enteric fever producing *Salmonella* species such as, *Salmonella* Typhi and *Salmonella* Paratyphi A were also identified but in relatively lower percentage. In India, 29.09% *Salmonella* Enteritidis and 1.5% *Salmonella* Typhimurium was observed in egg shell [27,28]. In the present study, serotype could not be identified in 2 (14.29%) *Salmonella* from egg shell detected by genus specific PCR. These negative findings might be due to the fact that we did not use all primers of other *Salmonella* [29].

Sensitivity and specificity of PCR in detecting *Salmonella species* in the present study is similar to other study [30]. In this study, one *Salmonella* strain isolated by culture was negative in PCR. The reason of such negative PCR result in isolated *Salmonella* might be due to the fact that *invA* gene was detected in PCR in this study to detect *Salmonella* and these culture positive *Salmonella* strains might have *invB or himA* gene [31] and PCR used in this study could not detect these genes. The higher sensitivity of PCR than culture is due to culture needs live bacteria but PCR can detect DNA of both live and dead bacteria [32].

Ciprofloxacin, combination of amoxicillin and clavulanic acid and imipenem are usually being used to treat ESBL producing gram negative bacteria in Bangladesh. But bacteria is developing resistance to these antibiotics rapidly which is evidenced by the fact that 84.62% bacteria were susceptible to imipenem in the present study and 100% bacteria other than *Salmonella* were resistant to amoxicillin plus clavulanic acid and ciprofloxacin. The reason of development of resistance to these drugs might be due to the fact that antibiotics are sold over the counter in Bangladesh and anybody can buy it without physician's prescription and in most of the cases they discontinue after partial cure. Now colistin is the most effective drug to treat these drug resistant bacteria which was effective against 100% isolated bacteria. On the other hand, 100% *Salmonella* serotypes were sensitive to chloramphenicol, ciprofloxacin and ceftiraxone in the present study. In Bangladesh ceftriaxone resistant *Salmonella* Typhi and Paratyphi has not yet been reported from human cases [33].

CONCLUSION

Eggs may be a source of transmission of different gram negative bacteria from market to the community. PCR is the sensitive method to detect *Salmonella* in culture negative samples. Early detection and proper hygienic practice should be maintained in handling and marketing eggs by the farm handlers and retailers to prevent spread of infection of different gram negative bacteria including *Salmonella* to the community. Ceftriaxone, ciprofloxacin and chloramphenicol are the most effective drugs against *Salmonella* and colistin is the most effective drug against other gram negative bacteria isolated from market eggs.

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