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Detection of potential pathogenic aerobic bacteria from egg shell and egg contents of hen collected from poultry

Fardows J[1], Shamsuzzaman SM[2]

[1]Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh. [2]Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh.
Email: smszaman@yahoo.com

Abstract

This study was done to identify different pathogenic aerobic bacteria from egg shell and egg contents of hen. Egg shells and egg contents of 150 eggs collected from poultry were tested. Of 150 egg shells, 130 (86.67%) yielded growth of bacteria and 60 (40%) *Esch. coli*, 25 (16.67%) *Providencia rettgeri*, 5 (3.33%) *Providencia alkalifaciens*, 20 (13.33%) *Citrobacter freundii*, 10 (6.67%) *Salmonella spp*, 10 (6.67%) *Enterobacter aerogenes* were isolated. No bacteria were isolated from 150 egg contents. Total 14 (9.33%) *Salmonella spp.* from egg shells and 7 (4.67%) *Salmonella spp.* from egg contents were identified by PCR. Most of the identified serotypes were *Salmonella* Enteritidis (42.86% from egg shells and 71.43% from egg contents). All (100%) *Salmonella* Typhi and *Salmonella* Paratyphi A were sensitive to ciprofloxacin and ceftriaxone.

Key words: aerobic bacteria, *Salmonella*, poultry eggs, culture, PCR.

Introduction

Poultry products especially eggs and egg products are nutritive food items and a vital constituent of human food in the world.[1] Inaccurately treated eggs can cause food-borne illness and it is a major public health problem and the main cause of diarrheal diseases affecting all developed and developing countries.[2] The absence of standard structures and drainage system in the poultry and relatively high humidity could have contributed to the high microbial growth.[3] Eggs have natural defense system against the contaminating microbes, such as cuticle, calcium hard shell, shell membrane and some antibacterial factors.[4] In spite of these, it can be contaminated with different food borne pathogen such as *Salmonella spp., Esch coli, Listeria monocytogens, Campylobacter jejuni, Proteus spp.* and *Klebsiella spp.*[3] It can be contaminated during formation and laying process.[5] Eggs have more possibility to become infected than fresh eggs due to the degradation of natural defense mechanisms in egg. Bacterial contamination can happen at three main parts of egg (egg yolk, albumen and shell membrane / egg shell).[6] *Salmonella* Enteritidis is able to invade the cells of the follicles before ovulation.[7]

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.[8] Other than enteric fever, the majority of infections results in asymptomatic or self-limited disease; however, in immuno-compromised patients, neonates and elderly, it requires antibiotic treatment.[9] The use of antibiotics in animals disrupts normal flora of intestine, resulting in to emergence of antibiotic-resistant Salmonella and their prolonged fecal shedding into the environment. Recently multi-drug resistant (MDR) strains have emerged, presumably due to the extensive use of antibiotic in veterinary practice.[10]

Salmonella isolation by conventional culture methods are based on pre-enrichment, enrichment and plating on selective and differential media and suspected colonies are then confirmed by biochemical and serological methods.[9,11] Generally these techniques take longer time and they give only presumptive results.[9] PCR for gene amplification has made it possible to detect low numbers of infectious agents.[12] One study has been done in Bangladesh regarding Salmonella

isolation by culture in 2011[9] and one study has been done regarding *Salmonella* identification by PCR in 2012[11] but only *Salmonella* Typhimurium has been identified. No study yet has been carried out regarding identification of other serotypes of *Salmonella* in Bangladesh. The specific objectives of this study are detection of different *Salmonella* serotypes from egg shells 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 done in the department of Microbiology, Dhaka Medical College (DMC), Dhaka, Bangladesh from July, 2012 to June, 2013. This protocol was approved by the Research Review Committee of the department of Microbiology of DMC and ethical clearance was obtained from the Ethical Review Committee of DMC. Oral consent was taken from poultry farm handlers before collecting eggs.

A total 150 poultry eggs were collected and both egg shells and egg contents were tested. Clean, undamaged eggs were included and focally contaminated and cracked eggs were excluded from this study. Eggs were collected directly from poultry farms in sterile containers and transported to the Microbiology laboratory of Dhaka Medical College with minimum delay.

Egg shell and egg contents processing, isolation and identification of organisms, serotyping of *Salmonella*, ESBL detection and antimicrobial sensitivity testing were done according to standard protocol.

Polymerase chain reaction (PCR): PCR was done conventionally and primers used for different serotype of *Salmonella* are listed in Table I.

Result

Among the 150 shells of eggs collected from poultry, 130 (86.67%) yielded growth of different bacteria and none of the 150 egg contents yielded growth. Among the aerobic bacteria isolated from egg shells, *Esch. coli* was the most common organism (40%) and 10 (6.67%) were *Salmonella* spp. (Table II).

Total 14 (9.33%) of the 150 egg shells and 7 (4.67%) of the 150 egg contents were positive for *Salmonella* by PCR. *Salmonella* Enteritidis was the most common *Salmonella* serotype detected from egg shells and egg contents by PCR (Table III and Fig 1).

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

Name	Genes	primers	Sequence (5'-3')	Base pair
Salmonella spp.	*invA*[13]	fliC-s	F-ATAGCCATCTTTACCAGTTCCCCC	284 bp
		fliC-as	R-GCTGCAACTGTTACAGGAATATGCC	
Salmonella Typhimurium	*fliC*[13]	fliC-s	F- ATAGCCATCTTTACCAGTTCCCCC	183 bp
		fliC-as	R- GCTGCAACTGTTACAGGAATATGCC	
Salmonella Enteritidis	*sefA*[14]	SEFA2	F-GCAGCGGTTACTATTGCAGC	310 bp
		SEFA4	R-TGTACAGGGACATTTAGCG	
Salmonella Typhi (O antigen)	*Tyv*[15]	tyv-s	F-GAGGAAGGGAAATGAAGCTTTT	615 bp
		tyv-as	R- TAGCAAACTGTCTCCCACCATAC	
Salmonella Paratyphi A (O antigen)	*Prt*[15]	parat-s	F-CTTGCTATGGAAGACATAACGAAC	258 bp
		parat-as	R-CGTCTCCATCAAAAGCTCCATAGA	
Salmonella Typhi (H antigen)	*fliC*[15]	fliCcom-s	F-AATCAACAACAACCTGCAGCG	750 bp
		fliCd-as	R-GCATAGCCACCATCAATAACC	
Salmonella Paratyphi A (H antigen)	*fliC*[15]	fliCcom-s	F-AATCAACAACAACCTGCAGCG	329 bp
		fliCa-as	R-TAGTGCTTAATGTAGCCGAAGG	

Table II: Frequencies of microbial isolates from egg shells among poultry eggs (n=150).

Types of isolates	n (%)
Esch. coli	60 (40.00)
Providencia rettgeri	25 (16.67)
Providencia alkalifaciens	5 (3.33)
Citrobacter freundii	20 (13.33)
Enterobacter aerogenes	10 (6.67)
Salmonella Typhi	1 (0.67)
Salmonella Paratyphi A	1 (0.67)
Others serotypes of *Salmonella*	8 (5.33)
Total	130 (86.67)

Of the 150 egg shells, 8 (80%) were positive by both culture and PCR. Six (4.29%) were positive by PCR but negative by culture and 2 (20%) was negative by PCR but positive by culture (Table-IV). Considering culture as gold standard, the sensitivity of PCR was 80%, specificity was 95.71%, positive predictive value was 57.14%, negative predictive value was 98.53% and accuracy was 94.67%. The difference in positivity between culture and PCR was statistically significant ($p<0.001$).

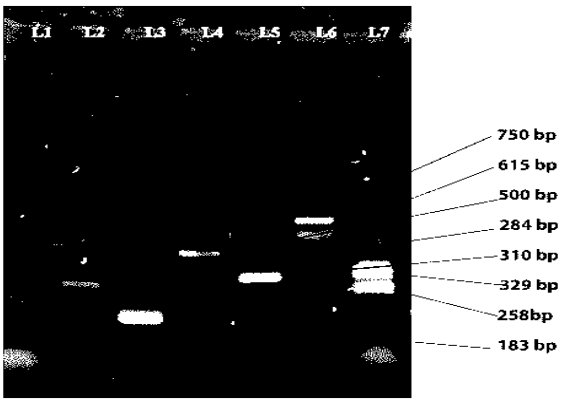

Fig. 1: Photograph of amplified DNA of different serotypes of *Salmonella*. Negative control *Esch. coli* ATCC 25922 (lane 1). Amplified DNA of 284 bp for *invA* gene of *Salmonella spp.* (lane 2), 183 bp for *fliC* gene of *S.* Typhimurium (lane 3), 310 bp for *sefA* gene of *S.* Enteritidis (lane 5),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

In the antimicrobial resistance pattern of *Salmonella*, no serotype was resistant to chloramphenicol, imipenem and gentamicin. The 2 (50%) isolated *Salmonella* Typhimurium were resistant to nalidixic acid and one (25%) to ciprofloxacin and ceftriaxone. All the isolated *Salmonella* Typhi and *Salmonella* Paratyphi A were sensitive to most of the antibiotics (Table-V).

Discussion

In this study, among the eggs collected from poultry farms, 86.67% egg shells and no egg contents yielded growth of pathogenic bacteria. In Iran 68.28% egg shells and in Thailand, 96.3% eggs collected from poultry yielded growth of pathogenic bacteria.[2,16] These bacterial contaminations might be from cloths and hands of poultry workers, use of same tray, environment, weather condition of the poultry.[3] In the developing countries specially Bangladesh, there are many poultry farms and inadequate refrigeration even no refrigeration, improper handling can increase the percentage of different bacterial contamination on egg shell.

Among the total isolated aerobic bacteria, 40% were *Esch. coli* (Table II). In India, relatively lower percentage of aerobic bacteria (28.74%) was observed[17] and in Iran, only 9% *Esch. coli* was reported.[5] Though *Esch. coli* is a normal inhabitant of intestinal tract of birds and it is of low risk for people but chickens are susceptible to colonization with *Esch. coli* O157:H7, an important Shiga toxin-producing, enterohemorrhagic pathogen for human.[5] In addition, 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. In this study, however, attempt to detect these diarrhoeagenic strains was not made. In addition to other gram negative bacteria, 10 (6.67%) *Salmonella spp.* were isolated. Enteric fever is endemic in many developing countries particularly Indian subcontinent including Bangladesh.[18] It is known to all that *Salmonella* transmission occurs mainly by food and drink. So, egg might be an important source of *Salmonella* transmission. Few studies in Bangladesh reported 8% - 12% *Salmonella* from eggs, however, prevalence of different *Salmonella* serotype was not reported in those studies.[9,11]

Table III: Identification of different Salmonella serotypes by PCR among Salmonella DNA positive sample from egg shells (n=14) and egg contents (n = 7)

Salmonella serotypes	Egg shells Positive n (%)	Egg contents Positive n (%)
S. Enteritidis	6 (42.86)	5 (71.43)
S. Typhimurium	4 (28.57)	-
S. Typhi	1 (7.14)	-
S. Paratyphi A	1 (7.14)	-
Unidentified *Salmonella*	2 (14.29)	2 (28.57)
Total	14 (100.00)	7 (100.00)

In the present study, 9.33% egg shells were positive for *Salmonella* by PCR by genus specific primer; 42.86% of the PCR detected *Salmonella* Were *Salmonella* Enteritidis and 28.57% were *Salmonella* Typhimurium (Table III). In this study, *Salmonella* Typhi and *Salmonella* Paratyphi A were identified but in relatively lower percentage. In India, 29.09% *S.* Enteritidis and 1.5% *S.* Typhimurium was observed in egg shell.[19,20] In this study, 7 (2.33%) *Salmonella* were detected by PCR from egg contents and most were *S.* Enteritidis (71.42%). *Salmonella* Enteritidis is the most frequently reported serovar from the egg shell as well as egg contents.[21,22] In chicken it has been shown that both *Salmonella* Typhimurium and *Salmonella* Enteritidis infect the reproductive

tract and contaminate eggs but *Salmonella* Enteritidis persists after eggs are laid.23 It has been proved that a specific gene possibly alters *Salmonella* Enteritidis interaction with egg albumin components, but *Salmonella* Typhimurium does not have this protective gene.[24] In the present study, serotype could not be identified in 2 (14.29%) *Salmonella* from egg shell and 2 (28.57%) from egg contents by PCR.

Table IV: Comparison between results of culture and PCR for *Salmonella spp.*

PCR	Culture		
	Positive n (%)	Negative n (%)	Total n (%)
Positive	8 (80.00)	6 (4.29)	14 (9.33)
Negative	2 (20.00)	134 (95.71)	136 (90.67)
Total	10 (100.00)	140 (100.00)	150 (100.00)

χ^2 = 39.0, df=1, p<0.001. The difference in positivity between culture and PCR was statistically significant.

These negative findings might be due to the fact that we did not use all primers of other *Salmonella* and these unidentified *Salmonella* might also be *Salmonella* Enteritidis or *Salmonella* Typhimurium but other phage type.[25]

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

Antibiotics	*S.* Enteritidis (n=6)	*S.*Typhimurium (n=4)	*S.* Typhi (n=1)	*S.* Paratyphi A (n=1)
Chloramphenicol	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nalidixic acid	5 (83.3)	2 (50.0)	0 (0.0)	0 (0.0)
Imipenem	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Amikacin	3 (50.0)	2 (50.0)	1 (100)	1 (100)
Ciprofloxacin	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)
Gentamicin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ceftriaxone	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)
Azithromycin	2 (33.3)	1 (25.0)	0 (0.0)	0 (0.0)
Amoxycillin + Clavulanic acid)	5 (83.3)	2(50.0)	0 (0.0)	0 (0.0)

In this study, of the 14 *Salmonella* detected by PCR (Table IV), 10 were positive by culture (p<0.001) and the sensitivity and specificity of PCR were similar to other study.[26]

Two *Salmonella* strains isolated by culture were negative in PCR even after repeated attempts of DNA extraction from the stored specimen and annealing. 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 and PCR could not detect these genes.[26] 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 and even presence of single DNA can be amplified and can be detected by PCR.[13]

In this study, all (100%) *Salmonella* Typhi and *Salmonella* Paratyphi A were sensitive to ciprofloxacin, ceftriaxone and chloramphenicol (Table V). In human case, these are the effective drugs and widely used against *Salmonella* Typhi and *Salmonella* Paratyphi.[27] But for the last few years' sensitivity of *Salmonella* to ciprofloxacin has been decreased and so far no ceftriaxone resistant *Salmonella* Typhi and *Salmonella* Paratyphi has been reported from human infection in Bangladesh. Multidrug resistant *Salmonella* Typhimurium was also reported from egg shell in Bangladesh.[28] Although, no data was found that *Salmonella* Typhimurium is resistant to ceftriaxone but in the present study, one (25%) *Salmonella* typhimurium were resistant to ceftriaxone which may give us cautionary signal regarding antibiotic resistance for *Salmonella* in future.

Conclusion: Eggs may be a source of transmission of different gram negative bacteria specially *Salmonella* and diarrhoeagenic *Esch. coli* from poultry to the community. Current study reflects that PCR is the sensitive method to detect *Salmonella* in culture negative samples. Present study also reflects that ceftriaxone, ciprofloxacin and chloramphenicol are the most effective drugs against *Salmonella* isolated from poultry eggs.

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