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Isolation and identification of *Escherichia coli* and *Salmonella* from poultry litter and feed

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ABSTRACT

The research work was conducted to detect and characterize of *Escherichia coli* and *Salmonella* found in the poultry feed and litter. A total of 48 samples were collected from the different Broiler farms of Dinajpur district and brought to the laboratory. Samples were propagated in nutrient broth and nutrient agar media followed by culture on selective media– Eosin Methylene Blue Agar, MacConley agae, Brilliant Green Agar, *Salmonella*-Shigella Agar. Biochemical properties of the isolates were studied and reaction in TSI agar slant was also observed. Gram's staining techniques were performed. All the isolates those were found to have the specific morphological, cultural characteristics and were selected for biochemical test and microscopic examination after staining. The overall prevalence of *E. coli* and *Salmonella* spp. in 4 poultry farm were recorded 62.50 % and 49.91 % respectively. In feed sample the prevalence of *E. coli* was 37.50% whereas in litter sample it was 87.50%. The prevalence of *Salmonella* in feed sample was 29.16% and in litter sample 66.66%. The results suggest that poultry litter is the major source of both *E. coli* and *Salmonella* spp for birds reared in litter system. The presence of *Salmonella* at the recorded rate in both the feed and litter pose significant alarming for the public health issue if not maintain proper hygienic steps in place.

Key words: *E. coli*, *Salmonella*, poultry litter and feed

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INTRODUCTION

Bangladesh is an agricultural country with a large number of domestic chickens and ducks. About 80% of the total population is living in the 68,000 villages of Bangladesh, and almost each and every village home holds 6-7 chickens. It is estimated that there are about 153 million chickens in Bangladesh (Samad, 2005). There are about one lakh poultry farms in Bangladesh, of which 20% rearing 1000 to 50,000 birds and remaining 80% are small in size with 100 to 1000 birds. Currently there are about 130 hatcheries (65 hatcheries with breeding farm) in Bangladesh, with two million broilers and 0.3 million layer parent stock which producing 4 to 5 million commercial day-old-chicks per week. The commercial broiler and layer farms are supplying about 0.2 million metric ton of poultry meat and 5210 million table eggs per year in Bangladesh (Samad, 2005).

The advancement of poultry industry in Bangladesh is interrupted by a number of constraints, of which major one is outbreak of disease causing about 30% mortality of chickens in every year (Ali 2004). The major etiological agents are the microorganisms, parasites, management causes, environmental causes and deficiency of mineral and vitamins. The major causes of microorganisms are *Escherichia coli*, *Salmonella*, fungus etc. However, the pathogens discharged from the chicken contaminate the litter,

feed, water and thus the nearby birds. The rapid growth of the poultry industry has resulted in the production of massive quantities of poultry wastes. These materials are alternatively viewed as essential soil fertilizers, energetic and nutritive substrates for feeds for animals (Smith, 1974; Jeffrey et al., 1998) but source of infection by pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* and *Campylobacter* spp (Martin et al., 1998). It is important to know the prevalence and distribution of different bacterial flora in poultry and its environment as many of them may be potential pathogen for poultry. Such information is also required to take necessary actions for the prevention and control of diseases caused by bacterial pathogens. The present study was undertaken to indentify and characterize the bacterial pathogens present in poultry litter as source of potential contaminants in poultry industry.

MATERIALS AND METHODS

Collection of samples

Samples (litter and feed) were collected from. Litter samples (10gm each) were randomly collected from the four commercial broiler farms with birds of 15 to 20 days old at Dinajpur district in Bangladesh and feed samples (10gm each) were directly taken from feeding trap of that farms. The total of 48 samples were collected from the broiler farms (Israful Poultry Farm, Basherhat; Harun Poultry Farm, Basherhat; Tarikul Poultry Farm, Basherhat; Vai-Vai Poultry Farm Kornai, Katapara) with at least 6 liter and 6 feed sample from each farm. Samples were collected aseptically and transferred immediately into a sterile petridish.The samples were then brought to the laboratory in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University. These samples were subjected to various bacteriological and biochemical examination in the laboratory.

Nutrient Broth (NB) and Nutrient Agar (NA) were used to grow the organisms from the collected samples before performing biochemical test and disinfectant efficacy test according to the procedure describe by Cheesebrough (1984). Eosin Methylene Blue (EMB) agar medium was used for the purpose of observing growth of *E. coli* (Cheesebrough, 1984). MC medium was used for culturing the organisms under the family Enterobacteriaceae (Cheesebrough. 1984). SS agar medium was used as a selective medium for *Salmonella* organism which causes enhancement of the growth of *Salmonella* while inhibiting the growth of other contaminating organisms and shows typical colony characters (Cheesebrough, 1984). Brilliant Green Agar (BGA) medium was used as a selective medium for the isolation and identification of *Salmonella* organisms (Cheesebrough, 1984).

Media

Nutrient broth, Motility Indole Urea (MIU) medium (HiMedia, India), Nutrient Agar (NA) medium (HiMedia, India), Eosin Methylene Blue (EMB) Agar (HiMedia, India), MacConkey's (MC) Agar medium (HiMedia, India), *Salmonella*-Shigella (SS) Agar (HiMedia, India), Brilliant Green Agar (BGA) (HiMedia, India), Triple Sugar Iron (TSI) Agar slant (HiMedia, India) were purchased as powdered form and prepared the media according to the manufactured instruction.

Reagents and solution

Methyl Red-Voges Proskauer broth (MR), Voges-Poskauer solution (VP), Crystal violet, Gram's iodine, Acetone alcohol and Safranine, Kovac's reagent, Ethyl alcohol (70% and 95%), Alpha naphthol solution, Potassium hydroxide solution, Phosphate Buffered Saline (PBS).

Preparation of inoculums

Each sample collected in sterile plastic bag was diluted with sterile phosphate buffered saline (PBS) and kept for 1 hour. Then 1 ml of sample was incubated into 9 ml of nutrient broth for enrichment and incubated overnight at 37oC.

Enrichment of samples

Immediately after collection of the samples were enriched in nutrient broth and incubated at 370C for overnight.

Isolation of bacteria

After enrichment in nutrient broth a small amount of inoculums from NB was streaked onto SS agar, BG agar, MC agar and TSI agar. The inoculated plates were incubated at 37 0C for overnight. After isolation of the organism on the selective media, differential screening media as TSI agar was used for further characterization. The test organisms were cultured into TSI agar slant by stab streak method. In TSI agar slant, if the organism ferments only glucose then the tube will turn yellow within a few hours. If the organism ferments lactose and/ or sucrose the slant will turn yellow and remain yellow for several days due to the increased level of acid production. Gas production of the organism can be ascertained by the appearance of bubbles in the agar. TSI agar can also be used to indicate whether Hydrogen sulphide (H_2S) has been produced which will appear as a black precipitate. Yellow slant, yellow butt, presence of gas bubbles and absence of black precipitate in the butt was positive for *E. coli*. The slant was pinked and butt was yellow and black for *Salmonella*.

Identification of bacteria

Colonial morphology

Colonial morphology such as shape, size, surface texture, edge and elevation, color and opacity developed after 24hrs of incubation in different media were carefully studied and recorded.

Biochemical tests

Isolated organism showing characteristic colonial morphology of *E. coli* and *Salmonella* on BA, BG, SS, MC, TSI and EMB agar were subjected to biochemical tests such as; sugar fermentation test, MR-VP test and Indol tests. Standard methods were followed to conduct these tests (Cowan, 1985).

Gram's staining

The representative *E. coli* and *Salmonella* colonies were characterized microscopically using Gram's stain according to the method described by Merchant and Packer, (1967). Briefly, a small colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violet solution was added on the smear for two minutes and then washed with running water. Lugol's iodine was added for one minute and then washed with running water. Acetone alcohol was added for few seconds. After washing with water, safranine was added for two minutes. The slide was then washed with water, blotted and dried in air and then examined under microscope with high power objectives 100X using immersion oil.

Maintenance of stock culture for bacteria

The stock culture was maintained following the procedures of Chowdhury et al. (1994). Pure culture of the isolated organisms were stored in sterilized 80% glycerin and used as stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 4°C in refrigerator for future use.

RESULTS AND DISCUSSION

Isolation and identification of *E. coli*

Cultural prevalence of E. coli in selected Poultry Farm

Table 1
Cultural prevalence of *E. coli* in selected Poultry Farm

No. of farms	Type of sample	No. of samples	Positive fo *E. coli*	Percentage
4	Feed	24	9	37.50
	Litter	24	21	87.50
Total		48	30	62.50

E. coli were isolated and identified from the samples after cultivation on NA, EMB agar and MC agar. The prevalence of *E. coli* in that study was 62.5% (table 1). Each of the positive samples were treated an isolate. The prevalence of *E. coli* in feed and litter samples were 37.50% and 87.50% respectively.

Identification of *E. coli* by different bacteriological methods

Table 2
Morphology, cultural characteristics and staining characteristics of isolated *E. coli*

Media used	Colony characteristics	Morphology (staining Characters)
Salmonella-Shigella agar	Slight pink smooth colony (Plate 5)	Gram-negative, pink color, small rod shaped appearance, arranged in single or paired short (Plate 10)
MacConkey's agar	Bright, pink colored transparent smooth raised colony (Plate 4)	
Eosin methylene blue agar	Yellow green characteristic metallic sheen (Plate 3)	
Nutrient agar	Circular, smooth, colorless colonies (Plate 2)	
Nutrient broth	Turbidity in the broth (Plate 1)	

(Plates 1 – 5 and 10 in supplementary materials)

Identification of *E. coli* was confirmed by colony characteristics at different bacteriological media presented in table 2.

Biochemical test for *E. coli*

Fermentation reaction with five basic sugars

All the isolates fermented the five basic sugars producing acid and gas. Acid production was indicated by the color change from reddish to yellow and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes. The Biochemical plates on TSI, MIU- indole test, MR and VP test are showed in Plate 6, 7, 8 and 9 respectively (plates 6- 9 in supplementary materials).

Table 3
Biochemical characteristics of *E. coli*

CHO fermentation and other biochemical tests		Result
Dextrose fermentation		+
Lactose fermentation		+
Sucrose fermentation		+
Mannitol fermentation		+
Indole production		+
MR test		+
VP test		-
TSI	Butt	Y
	Slant	Y
	H_2S	-
	Gas	+

Legends: + = Positive; - = Negative; MR= Methyl Red; VP= Voges-Proskauer; TSI = Triple sugar iron; Y= Yellow; R= Red.

Isolation and identification of *Salmonella* spp

Table 4
Cultural prevalence of *Salmonella* spp in selected Poultry Farm

No. of farms	Type of sample	No. of samples	Positive for *Salmonella*	Percentage
4	Feed	24	7	29.16
	Litter	24	16	66.66
Total		48	23	47.92

The overall prevalence of *Salmonella* spp in this study was 47.92 %. The prevalence of *Salmonella* in feed and litter were 29.16% and 66.66% respectively.

Isolation of Salmonella spp from selected Poltry Farm

Salmonella spp. were isolated and identified from the samples after cultivation on NA, MC agar, EMB agar, Simmons Citrate agar, SS agar, BGA medium.

Identification of Salmonella spp.

The results of morphological study and cultural characteristics are presented in table 5.

Table 5
Morphology, cultural and staining characteristics of isolated *Salmonella spp*

Media used	Colony characteristics	Morphology (staining Characters)
Salmonella-Shigella agar	Opaque translucent colorless smooth round colonies (Plate 11).	Gram negative short rod shaped singly arranged (Plate 19)
MacConkey's agar	Pale, Colorless, smooth, transparent, raised colonies (Plate 13).	
Eosin Methylene Blue agar	Pink color colonies (Plate 14)	
Brilliant green agar	Pale pink color colonies against a yellowish background (Plate 12).	
TSI agar	Transparent, smooth round colonies (Plate 15)	
Nutrient agar	Translucent, opaque, smooth colonies (Plate 2)	
Nutrient broth	Turbidity in the broth (Plate 1).	

Plate 1, 2, 13-15, 19 in supplementary materials

Biochemical test for *Salmonella* spp.

Fermentation reaction with five basic sugars

All the isolates fermented dextrose and mannitol and produced acid and gas or only acid. No fermentation was seen in lactose and sucrose but slight fermentation occurred in maltose. Acid production was indicated by the color change from reddish to yellow. The Biochemical plates on TSI, MIU-indole test, MR and VP test are showed in Plate 15, 16 17 and 18 respectively. (Plate 15-18 in supplementary materials).

The isolation, identification and characterization of the *E. coli* and *Salmonella* found in the feed and litter of commercial broilers were performed by using different cultural, biochemical test. The study will develop awareness about the biosecurity measures in poultry farm and help to prevent contamination of bird with *E. coli* and *Salmonella* from feed and litter.

Differences in colony morphology manifested by the isolates may be due to loosing or acquiring some properties by the transfer of host or choice of host tissue observed by Merchant and Packer (1967) and Nzouankeu et al. (2010).

Table 6
Biochemical characters of the *Salmonella* spp.

CHO fermentation and other biochemical tests		Result
Dextrose fermentation		+
Lactose fermentation		-
Sucrose fermentation		-
Mannitol fermentation		+
Indole production		-
MR test		+
VP test		-
TSI	Butt	Y
	Slant	R
	H_2S	+

Legends: + = Positive; - = Negative; MR= Methyl Red; VP= Voges-Proskauer; TSI = Triple sugar iron; Y= Yellow; R= Red.

In the present study the isolated *E. coli* organism fermented dextrose, maltose, lactose, sucrose and manitol with the production of both acid and gas. Results of MR, Indole test of the *E. coli* isolates were positive as reported by Buxton and Fraser 1977. In Gram's staining, the morphology of the isolated bacteria exhibited pink, small rod shape, Gram negative bacilli which was supported by several authors (Buxton and Fraser, 1977; Freeman 1985).

It was also found that the prevalence of *E. coli* infection (62.50 %) was higher than the *Salmonella* (47.92 %) in commercial broilers farm. Prevalence of *E. coli* and *Salmonella* spp. were

87.50% and 66.66% in litter where as 37.50% and 29.16% in feed respectively. The result evidenced by Ghanbarpour et al. (2011) where the prevalence of *E. coli* was 63.64 % which was also in close agreement with the findings of Mishra et al. (2002). However higher prevalenceof *E. coli* (71.80%) was observed by Derakhshantar and Ghanbarpour (2002) might be due to different environmental condition, managemental condition, food habit and mixed infection with other microbes.

In the present study, specific media and biochemical tests which were used for the detection of *Salmonella* spp were also similarly used by a number of scientists (Tiabaijuka et al. 2003; Zschock et al. 2000 and Merchant and Packer 1967). In this study the colony characteristics of *Salmonella* spp. observed on different media were similar to the findings of other authors. *Salmonella* isolates were able to ferment the five basic sugars by producing both acid and gas. However differentiation of *Salmonella* into species level was difficult based on their sugar fermentation pattern (Buxton and Fraser. 1977). All the isolates of *Salmonella* in this study fermented dextrose, maltose and mannitol and produced acid and gas but not fermented sucrose and lactose, some isolated organism produced hydrogen sulfide gas which satisfied the statement of Buxton and Fraser (1977).

It was found that prevalence of *Salmonella* spp infection in the study was 47.92 %. The result are evidenced by Ahmed et al., (2007), Habib-ur-Rehman et al. (2004) and Biswas et al. (2004) who studied the causes of *Salmonella* spp in the same environmental condition in India and Bangladesh.
Litter sample was the highest source of contamination of *E. coli* and *Salmonella* than that of feed sample. However the pathogenicity of identified *E. coli* and *Salmonella* in feed and litter should be determined. From the present study it could be concluded that the commercial broiler farms should be periodically cheeked for the presence of pathogens and the prevention of microbial infections and biosecurity plan to the farms should be taken accordingly.

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