SHORT COMMUNICATION

Isolation and molecular-based identification of bacteria from unhatched leftover eggs of ducks in selected mini-hatcheries of Kishoreganj, Bangladesh

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ABSTRACT

Objectives: The study was designed for isolation and identification of the bacteria present in unhatched leftover eggs of duck in selected mini-hatcheries of Kishoreganj, Bangladesh.

Materials and Methods: A total of 54 unhatched discarded eggs were collected as samples from different mini-hatcheries of Tarail and Itna Upazilas of Kishoreganj and aseptically carried to the laboratory in the icebox. Surface washings \((n = 54)\) and inner contents \((n = 54)\) were collected and enriched in Luria–Bertani broth followed by the isolation of pure colonies of different bacteria onto eosin methylene blue agar, mannitol salt agar, Salmonella–Shigella agar, and blood agar plates. Identification of the bacterial isolates was done by cultural properties, staining, and biochemical tests followed by molecular detection by Polymerase chain reaction.

Results: Of 108 samples, 62 were found positive for Salmonella spp. \((76\%)\), 59 for E. coli \((54\%)\), 52 for Staphylococcus spp. \((48\%)\), and 5 for Clostridium spp. \((9\%)\). From the egg surface samples, Staphylococcus spp. were recovered in the highest \((67\%)\) followed by Salmonella spp. \((59\%)\), E. coli \((56\%)\), and Clostridium spp. \((9\%)\). From the inner contents of eggs, Salmonella spp. were recovered in the highest \((56\%)\), followed by E. coli \((53\%)\) and Staphylococcus spp. \((30\%)\).

Conclusion: The isolated bacteria might be associated with the decreased hatchability and embryo mortality in the mini-hatcheries of duck.

Introduction

In Bangladesh, duck occupies the second place next to chicken comprising about 16.52% \((55.85\text{ million})\) of the total poultry population \((337.998\text{ million})\) in the table egg production \([1]\). It has a significant contribution as a source of animal protein and generates employment opportunities for the farmers and landless women of the rural areas of the country \([2]\). About one-ninth of the total land of Bangladesh is low, providing ideal conditions for duck rearing. However, this sector could not flourish enough due to a lack of fertile eggs and the high cost of professional hatcheries. To mini-hatcheries is a small-scale incubator used to hatch duck eggs using low-cost traditional techniques. Nowadays, people are using different types of mini-hatcheries such as the rice husk method, quilt method, and sand method \([3]\). The hatchability in the mini-hatcheries ranges from 65% to 75% with considerable embryonic death \([4]\). The death of the embryo during incubation and egg hatching and vitality of newly hatched ducklings are influenced by the extent of bacterial contamination in poultry hatcheries, and it is established as one of the main factors \([5,6]\). Many authors have stated that the level of hygiene in the hatcheries has a relationship with the rate of embryonic death and the health status of newly hatched chicks. The risk of penetration through the eggshell by microorganisms such as Escherichia coli, Staphylococcus.
Materials and Methods

Collection of samples

The study period was between January and June 2019. The unhatched damaged eggs \((n = 54)\) were collected randomly from nine different mini-hatcheries of Tarail and Itna Upazillas of Kishoreganj district and carried to the laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh, Bangladesh. Surface swab \((n = 54)\) of each egg was obtained using sterile cotton buds. The outer shell was washed thoroughly with 2% tincture iodine, dried, mopped with 70% alcohol, and broken with the sterile blade for the collection of inner content \((n = 54)\) with or without dead embryos.

Isolation of bacteria

Luria–Bertani broth was used for the initial enrichment of the samples for 24 h at 37°C. A loop full of the enriched cultures was purposively streaked onto different bacteriological media including Salmonella–Shigella agar, eosin methylene blue agar, and mannitol salt agar and incubated at 37°C aerobically for 24 h. Blood agar plates were used for the isolation of anaerobes. After streaking a loop full of the enriched culture onto the media, it was incubated anaerobically using an anaerobic jar at 37°C until the pure culture was obtained.

Identification of the isolated bacteria

Colony characteristics such as size, shape, arrangement, elevation and edge, surface texture, opacity, and color developed on various selective media; microscopic observation after gram staining; and the results of different biochemical tests such as sugar fermentation test, methyl-red, Voges–Proskauer, indole, catalase, and motility tests were used as a basis for the identification of bacteria [10].

DNA preparation

The genomic DNA of the isolated bacteria was extracted by conventional boiling method following the protocol described by Rawool et al. [11].

Polymerase chain reaction (PCR)

PCR primers and conditions used in this study are provided in Table 1 with the expected product size. About 25 μl volume PCR reaction mixture was prepared with 12.5 μl 2× Master Mix (Promega, San Luis Obispo, CA), 1.0 μl of forward primer (10 pmol/μl), 1.0 μl of reverse primer (10 pmol/μl), 5.0 μl of DNA template, and 5.5 μl of deionized water. The separation of PCR product was done by gel electrophoresis using 1.5% agarose gel in 50× Tris-Acetic acid-EDTA (TAE) buffer. Visualization was performed using a ultraviolet (UV) transilluminator (Biometra, Germany) after staining with ethidium bromide (0.5 μg/ml).

Results and Discussion

Duck production both in commercial and household levels largely depends on the mini-hatcheries as it plays an essential role in collecting eggs from the farmers and selling newly hatched ducklings to commercial and household duck farmers. In this case, a significant problem is different types of hatchery-borne bacterial diseases, which play an essential role in lowering hatchability and decreased performance of offspring [12].

In general, bacteria contaminate eggs in two possible routes. First, at the time or after oviposition, penetration through the eggshell occurs from the contaminated feces [13,14] and, second, contaminates the eggshells or eggshell membranes, yolk, and albumen originating from the infected reproductive organs directly before oviposition [15,16]. These routes are a potential source of the pathogen, participating as the etiology of diseases such as omphalitis or infection of yolk sac, which are commonly responsible for death within 24 h of the birth of ducklings, with the highest survivability of 5–7 days [17]. Various bacteria may be involved in yolk sac infection, including E. coli, Staphylococci, Proteus, Clostridium, and Pseudomonas spp. [18].

In this study, four different species of bacteria, such as Salmonella spp., E. coli, Staphylococcus aureus, and Clostridium spp., were identified and isolated based on cultural, staining, biochemical, and molecular examinations (Table 2; Figs. 1–4). The results of isolation are in agreement with the findings of the previous studies [19–21]. The overall prevalence of Salmonella spp., E. coli, Staphylococcus aureus, and Clostridium spp. was found as 76%, 54%, 48%, and 9%, respectively. The prevalence is much higher than earlier reports [19,20], which might be attributed to the poor hygienic condition of the mini-hatcheries involved.
in this study. In most cases, an association of more than one bacterial species was reported. Bacteria were isolated, arranged in order of decreasing frequency by Al-Sadi et al. [21], which included *Escherichia coli*, *Staphylococcus* spp., and *Salmonella* spp. This study was also aimed to discriminate the prevalence of those mentioned above four bacterial species on the shell surface as well as in the inner contents. *Staphylococcus* spp. were recovered in the highest number (67%), followed by *Salmonella* spp. (59%), *E. coli* (56%), and *Clostridium* spp. (9%) on the shell surface. Conversely, the prevalence of *Salmonella* spp. was highest (56%) followed by *E. coli* (53%) and *Staphylococcus* spp. (30%) in the inner contents. The results of this study are comparable with the previous reports, which have reported a variable prevalence of the isolated bacterial species in the shell surface and inner contents of the eggs [22–27]. The differences between the studies might contribute to the duck

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**Table 1.** List of primers and PCR conditions used in this study.

| Primer name | Sequence (5’-3’) | Target Gene | Bacteria | PCR conditions | Product size | References |
|-------------|-----------------|-------------|----------|----------------|--------------|------------|
| invA F      | ATCAGTACCAGTCGCTTATCTTGAT | invA | *Salmonella* spp. | 94°C for 5 min; 29 cycles of 94°C for 30 sec, 52°C for 2 min, 72°C for 45 sec; final extension cycle at 72°C for 5 min | 211-bp | [28] |
| invA R      | TCTGTTCACCAGGCATTTACCAT | nuc | *Staphylococcus* spp. | 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 45 sec, 72°C for 1 min; final extension at 72°C for 10 min | 279-bp | [29] |
| S.ARS-F     | GCGATTGATGGTGATACCGT | nuc | *Staphylococcus* spp. | 95°C for 5 min; 30 cycles of 95°C for 45 sec, 52°C for 1 min; final extension at 72°C for 5 min | 585-bp | [30] |
| S.ARS-R     | AGCCAAGCCTGACGAAGCTAACG | nuc | *Staphylococcus* spp. | 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 45 sec, 72°C for 1 min; final extension at 72°C for 5 min | 585-bp | [30] |
| ECO-1       | GACCTCCTTTAGTTGACCAGA | 16SrDNA | *E. coli* | 95°C for 5 min; 30 cycles of 94°C for 45 sec, 52°C for 1 min; final extension at 72°C for 5 min | 585-bp | [30] |
| ECO-2       | CACAGCTGAGCTGACCA | 16SrDNA | *E. coli* | 95°C for 5 min; 30 cycles of 94°C for 45 sec, 52°C for 1 min; final extension at 72°C for 5 min | 585-bp | [30] |
| 16SrNAR     | GTGGACTACGGGTATCTAATCC | 16SrRNA | *Clostridium* spp. | 95°C for 5 min; 30 cycles of 94°C for 45 sec, 52°C for 1 min; final extension at 72°C for 5 min | 800-bp | [31] |

**Table 2.** Prevalence of isolated bacteria.

| Sample (egg) | Type of sample | Prevalence of bacteria (%) | E. coli | Salmonella spp. | Staphylococcus spp. | Clostridium spp. |
|--------------|----------------|---------------------------|--------|----------------|-------------------|-----------------|
| N = 54       | Egg surface swab (54) | 30 (56) | 32 (59) | 36 (67) | 5 (9) |
|              | Inner content (54) | 29 (53) | 30 (56) | 16 (30) | 0 |
| Total        | 108             | 59 (54) | 62 (76) | 52 (48) | 5 (9) |

**Figure 1.** Amplification of 16S rRNA of *E. coli* isolated from different duck hatcheries. Lane 1: 100-bp size DNA marker; lane 2: positive control; lane 3: negative control without DNA; and lanes 4–17: representative *E. coli* isolates.
rearing environments and housing system, management system, and biosecurity level of the hatchery, breeding site and practices, geographical area, and season.

Interestingly, no significant difference was found in the prevalence of the isolated bacteria in the shell as well as the inner contents of the eggs examined in this study, indicating that these bacteria might be originated from eggshell contamination. However, further studies are necessary to confirm the results as well as to determine whether they are resulted from fecal contamination of the eggshell surface or originated from the hatchery environment.
Conclusion

Of 108 samples, 62 were positive for *Salmonella* spp. (76%), 59 for *E. coli* (54%), 52 for *Staphylococcus* spp. (48%), and 5 for *Clostridium* spp. (9%). Some of these isolated bacteria might be associated with the decreased hatchability and embryonic mortality in the duck mini-hatcheries. Therefore, restricted hatchery sanitation, together with the use of suitable disinfectants, is recommended to minimize the risk of bacterial contamination and the possible related effect on hatchability. Besides, further studies are necessary to evaluate the virulence and association of the isolated bacteria with embryonic death and decreased hatchability.

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Conflict of interest

The authors declare no conflict of interest.

Authors’ contribution

The work was designed by Md. Shahidur Rahman Khan, Md. Shafigul Islam, and Saifur Rahman. Sadia Afrin Punom, Shyakya Tasnim Pritha, and Md. Muket Mahmud conducted the experiments. The first draft of this manuscript was prepared by Sadia Afrin Punom. Jayedul Hassan critically checked and improved the manuscript. Md. Shafigul Islam read and approved finally for publication.

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