JOURNAL OF ADVANCED VETERINARY AND ANIMAL RESEARCH
ISSN 2311-7710 (Electronic)
http://doi.org/10.5455/javar.2020.g406
A periodical of the Network for the Veterinarians of Bangladesh (BDvetNET)

March 2020
VOL 7, NO. 1, PAGES 164–169

SHORT COMMUNICATION

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

Sadia Afrin Punom, Md. Shahidur Rahman Khan, Shayka Tasnim Pritha, Jayedul Hassan, Saifur Rahman, Md. Muket Mahmud, Md. Shafiqul Islam
Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.

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.

ARTICLE HISTORY

Received October 26, 2019
Revised February 01, 2020
Accepted February 02, 2020
Published February 19, 2020

KEYWORDS

Bacteria, duck mini-hatchery, PCR, unhatched leftover eggs.

Introduction

In Bangladesh, duck occupies the second place next to chicken comprising about 16.52% (55.85 million) of the total poultry population (337.998 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 minimize the cost and meet up the demand of ducklings, farmers from many corners of Bangladesh have started hatching eggs employing mini-hatchery technologies.

Mini-hatchery 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*

Correspondence Md. Shafiqul Islam ✉ shafiqdvm@yahoo.com Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.

How to cite: Punom SA, Khan MSR, Pritha ST, Hassan J, Rahman S, Mahmud MM, Islam MS. Isolation and molecular-based identification of bacteria from unhatched leftover eggs of ducks in selected mini-hatcheries of Kishoreganj, Bangladesh. J Adv Vet Anim Res 2020; 7(1):164–9.

aureus, Clostridium spp., *Bacillus* spp., *Pseudomonas* spp., *Aspergillus* spp., and so on is increased by hatchery waste such as debris and fluff of eggshell, infertile eggs, culled dead embryos and chicks, fluids from cracked eggs, and the overall poor hygienic management in the hatcheries which can result in increased rates of embryonic death and decreased rates of viability of newly hatched ducklings [7–9]. Although, in Bangladesh, few research works had been conducted on the potentiality, productivity, and profitability of duck rearing and mini-hatcheries, the association of bacterial infection with decreased hatchability or embryonic death has not been studied yet. Therefore, the present research was undertaken with a view to isolate and identify the bacterial pathogens that were important and present in the unhatched leftover eggs in the duck mini-hatcheries.

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, arrangements, 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* spp., 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

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

Primer name	Sequence (5'-3')	Target		PCR conditions	Product size	References
		Gene	Bacteria			
invA F	ATCAGTACCAGTCGTCTTATCTTGAT	*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	TCTGTTTACCGGGCATACCAT					
S.ARS-F	GCGATTGATGGTGATACGGT	*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-R	AGCCAAGCCTTGACGAACTAAAGC					
ECO-1	GACCTCGGTTTAGTTCACAGA	16SrDNA	*E. coli*	95°C for 5 min; 30 cycles of 94°C for 45 sec, 52°C for 45 sec, 72°C for 1 min; final extension at 72°C for 5 min	585-bp	[30]
ECO-2	CACACGCTGACGCTGACCA					
16SrRNAF	GAGAGTTTGATCCTGGCTCAG	16SrRNA	*Clostridium* spp.	95°C for 5 min; 32 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min; final extension at 72°C for 10 min	800-bp	[31]
16SrRNAR	GTGGACTACCAGGGTATCTAATCC					

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)

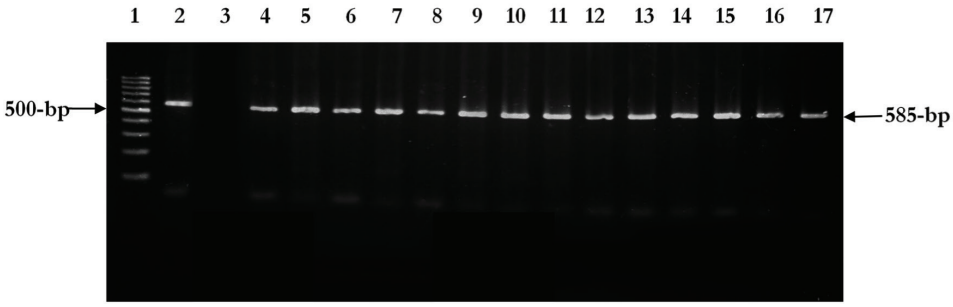

Figure 1. Amplification of 16S rRNA of *E. coli* isolated from different duck hatcheries. Lane 1: 100-bp size DNA marker; lane 2: positive control; lane 3: negative control without DNA; and lanes 4–17: representative *E. coli* isolates.

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

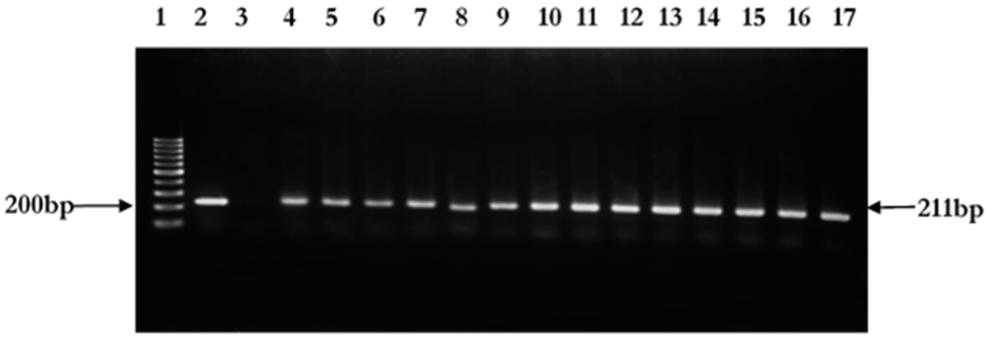

Figure 2. Amplification of *InvA* gene of *Salmonella* spp. isolated from different hatcheries. Lane 1: 100-bp size DNA marker; lane 2: positive control; lane 3: negative control without DNA; and lanes 4–17: representative *Salmonella* isolates.

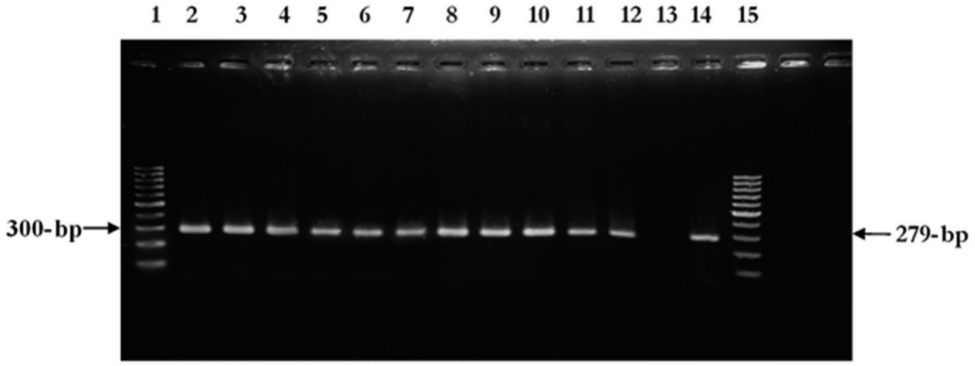

Figure 3. Amplification of *nuc* gene of *S. aureus*. Lanes 1 and 15: 100-bp size DNA marker; lanes 2–12: representative *S. aureus*; lane 13: negative control without DNA; lane 14: positive control.

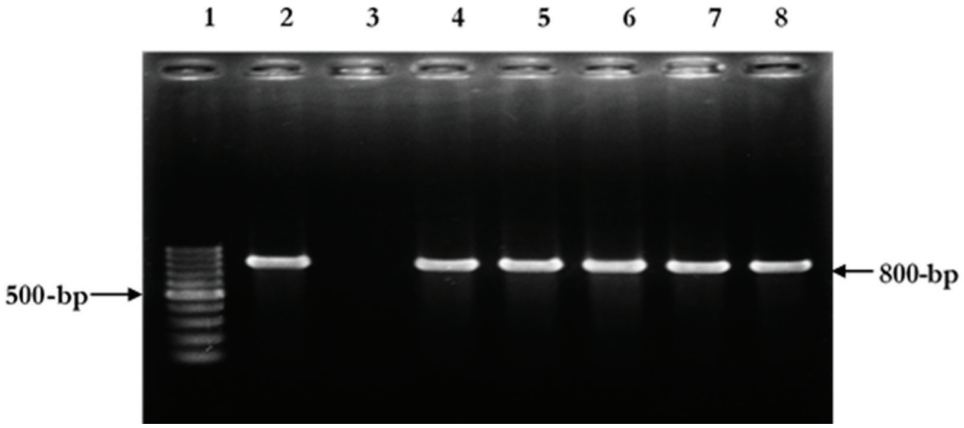

Figure 4. Amplification of the 16S rDNA gene. Lane 1: 100-bp size DNA marker; lane 2: positive control; lane 3: negative control without DNA; and lanes 4–8: 16S rDNA-positive *Clostridium* spp.

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.

Acknowledgment

Authors are grateful to the Ministry of Science and Technology, Government of People's Republic of Bangladesh, for funding the research and also to the Department of Microbiology and Hygiene, Bangladesh Agricultural University, for technical support.

Conflict of interest

The authors declare no conflict of interest.

Authors' contribution

The work was designed by Md. Shahidur Rahman Khan, Md. Shafiqul Islam, and Saifur Rahman. Sadia Afrin Punom, Shayka 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. Shafiqul Islam read and approved finally for publication.

References

[1] Department of Livestock Services (DLS). Livestock Economy at a Glance 2017-2018: Livestock Economics Section, Ministry of Fisheries and Livestock, Government of the People's Republic of Bangladesh, Dhaka, Bangladesh.

[2] Alam M, Uddin A, Bablu M, Kamaly M, Rahaman M. Socio-economic profile of duck farmers and duck management practices in Rajshahi region. Bangladesh J Anim Sci 2013; 41(2):96–105; http://dx.doi.org/10.3329/bjas.v41i2.14124

[3] Hassan J. Mini-hatchery: a low cost hatching technology. BDvet iNewsl 2011:1(2).

[4] Makaremuzzaman M, Hasnath MR, Miah MY, Belal SA, Hasan MK. Study on rice husk incubation for ducklings production in Sunamganj District of Bangladesh. Int J Agricul Biosci 2016; 5(5):291–5.

[5] Mauldin J. Reducing contamination of hatching eggs. Technical article. Poultry Industry, 2008. https://en.engormix.com/poultry-industry/articles/contamination-of-hatching-eggs-t34195.htm (Accessed on October 25, 2020)

[6] Qureshi AA. Hatchery sanitation and chick mortality. World Poult 2002; 24–5.

[7] Amer MM, ELbayoumi KM, Amin Girh ZM, Mekky HM, Rabie NS. A study on bacterial contamination of dead in shell chicken embryos and culled one day chicks. Int J Pharm Phytopharmacol Res 2017; 7(2):5–11.

[8] Gehan ZM. A new approach to evaluate the hygienic condition of commercial Hatcheris. Int J Poult Sci 2009; 8(11):1047–51; http://dx.doi.org/10.3923/ijps.2009.1047.1051

[9] Sheldon BW, Brake J. Hydrogen peroxide as an alternative hatching egg disinfectant. Poult Sci 1991; 70(5):1092–8; http://dx.doi.org/10.3382/ps.0701092

[10] Cheesbrough M. Medical laboratory manual for tropical countries. 1st ed. English Language Book Society, London, UK, pp. 400-80, 1985.

[11] Rawool DB, Malik SVS, Barduddhe SB, Shakunnatala I, Aurora R. A multiplex PCR for detection of virulence associate genes in Listeria monocytogenes. Int J Food Saf 2007; 9:56–62.

[12] Cadirci S. Disinfection of hatching eggs by formaldehyde fumigation- a review Arch. Geflügelk 2009; 73(2):116–23.

[13] Messens W, Grijspeerdt K, Herman L. Eggshell penetration by *Salmonella*: a review. World's Poult Sci J 2005; 61(1):71–86; http://dx.doi.org/10.1079/wps200443

[14] De Reu K, Grijspeerdt K, Messens W, Heyndrickx M, Uyttendaele M, Debevere J. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella enteritidis*. Int J Food Microbiol 2006; 112(3):253–60; http://dx.doi.org/10.1016/j.ijfoodmicro.2006.04.011

[15] Okamura M, Kamijima Y, Miyamoto T, Tani H, Sasai K, Baba E. Differences among six *Salmonella* serovars in abilities to colonize reproductive organs and to contaminate eggs in laying hens. Avian Dis 2001; 45(1):61; http://dx.doi.org/10.2307/1593012

[16] Okamura M, Miyamoto T, Kamijima Y, Tani H, Sasai K, Baba E. Differences in abilities to colonize reproductive organs and to contaminate eggs in intravaginally inoculated hens and *in vitro* adherences to vaginal explants between *Salmonella enteritidis* and other *Salmonella* serovars. Avian Dis 2001; 45(4):962; http://dx.doi.org/10.2307/1592875

[17] Amare A, Amin AM, Shiferaw A, Nazir S, Negussie H. Yolk sac infection (Omphalitis) in Kombolcha poultry farm, Ethiopia. America-Eurasian J Sci Res 2013; 8(1):10–4.

[18] Cortes CR, Isaies GT, Cuello CL, Floes JMV, Anderson RC, Campos CE. Bacterial isolation rate from fertile eggs, hatching eggs and neonatal broilers with yolk sac infection. Rev Latinoam Microbiol Rev Latinoam Microbiol 2004; 46:12–6.

[19] Azmy RW. Some studies on bacterial agents causing embryonic mortalities in chickens and ducks. Department of Avian and Rabbit Medicine Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, pp. 80–4, 2010.

[20] Ciocîrlan E. Research on the incubation bacterial microflora. Cercetări Agronomice în Moldova 2008; 2(134).

[21] Al-Sadi HI, Basher HA, Ismail HK. Bacteriologic and pathologic studies on dead-in-shell chicken embryos. Iraqi J Vet Sci 2000; 13(2):297–307.

[22] Ema F, Arif M, Islam M, Khatun M. Isolation and identification of duck egg-borne bacteria and their antibiogram profile. J Advan Vet Anim Res 2018; 5(3):110; http://dx.doi.org/10.5455/javar.2018.e253

[23] Parveen A, Rahman MM, Fakhruzzaman M, Akter MR, Islam MS. Characterization of bacterial pathogens from egg shell, egg yolk, feed and air samples of poultry houses. Asian J Med Biol Res 2017; 3(2):168–74; http://dx.doi.org/10.3329/ajmbr.v3i2.33564

[24] Mahmud MS, Kabir ML, Alam SMS, Ali MM, Towhid ST. Prevalence of *Salmonella* spp. in poultry eggs from different retail markets at Savar area, Bangladesh. Am J Food Sci Health 2015; 1:27–31.

[25] Stępień-Pyśniak D. Occurrence of Gram-negative bacteria in hens' eggs depending on their source and storage conditions. Polish J Vet Sci 2010; 13(3):507.
[26] Raji MA, Kwaga JO, Bale JO, Henton M. Biochemical and serological characterization of *Escherichia coli* isolated from colibacillosis and dead-in-shell embryos in poultry in Zaria, Nigeria. Nigerian Vet J 2007; 27(2); http://dx.doi.org/10.4314/nvj.v27i2.3513
[27] Adesiyun A, Offiah N, Seepersadsingh N, Rodrigo S, Lashley V, Musai L. Microbial health risk posed by table eggs in Trinidad. Epidemiol Infect 2005; 133(6):1049; http://dx.doi.org/10.1017/s0950268805004565
[28] Ogunremi D, Nadin-Davis S, Dupras AA, Márquez IG, Omidi K, Pope L. Evaluation of a multiplex PCR assay for the identification of *Salmonella* serovars *Enteritidis* and *Typhimurium* using retail and abattoir samples. J Food Protect 2017; 80(2):295–301; http://dx.doi.org/10.4315/0362-028x.jfp-16-167
[29] Kalorey DR, Shanmugam Y, Kurkure NV, Chousalkar KK, Barbuddhe SB. PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. J Vet Sci 2007; 8(2):151; http://dx.doi.org/10.4142/jvs.2007.8.2.151
[30] Hassan J, Parvej MS, Rahman MB, Khan MSR, Rahman MT, Kamal T. Prevalence and characterization of *Escherichia coli* from rectal swab of apparently healthy cattle in Mymensingh, Bangladesh. Microb Health 2014; 3(1):12–4; http://dx.doi.org/10.3329/mh.v3i1.19775
[31] Dhalluin A, Lemée L, Pestel-Caron M, Mory F, Leluan G, Lemeland J-F. Genotypic differentiation of twelve *Clostridium* species by polymorphism analysis of the triosephosphate isomerase (tpi) gene. Syst Appl Microbiol 2003; 26(1):90–6; http://dx.doi.org/10.1078/072320203322337362