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≪Research Note≫

Isolation and Identification of Non-coliform Gram-negative Bacteria in Hatching Eggs to Evaluate the Effect of Egg Fumigation by Formaldehyde

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Non-coliform gram-negative bacteria like *Salmonella, pseudomonas and proteus* are the causes of yolk sac infection. In this study, we evaluated the effect of formaldehyde on the reduction rate of egg shell, yolk and yolk sac contamination.

Two hundred and forty hatching eggs from a broiler breeder farm and hatchery as well as sixty newly-hatched chicks (5 stages) were selected for bacteriological examinations.

Stages include: Stage 1: Before cleaning and first disinfection, Stage 2: After first disinfection, Stage 3: Before setting inside the setter, Stage 4: Time of transferring from setter to hatcher, Stage 5: Newly-hatched chicks.

Alcaligenes faecalis was isolated from 18.3%, 11.7%, 8.3% and 10.0% of egg shells from stages 1 to 4 respectively and it was also isolated from 11.7% of yolk sacs. Non-coliform gram-negative bacteria were not isolated from yolks in any stages. Based on this research the existence of non-coliform gram-negative bacteria on the hatching egg shells is normal. Immediately egg collection after laying and proper disinfection with formaldehyde can lead to a significant reduction of non-coliform gram-negative bacteria with which the risk of bacteria penetration in to the yolk will decrease dramatically. Formaldehyde is usually effective in reducing non-coliform gram-negative contamination, however, in this study it could not affect significantly ($p=0.323$) on these contaminations, which maybe due to the fact that the bacteria were in the form of spores. The reduction of contamination rate was significant ($p=0.049$) only between stages 1and 3 which can be attributed to secondary fumigation by formaldehyde.

Key words: formaldehyde, hatching eggs, isolation, non-coliform bacteria

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Introduction

Yolk sac infection is a common disease at first week of age in chickens. This infection often occurs as a result of egg shell contamination (Board *et al.,* 1964; Lifshitz *et al.,* 1964). Yolk sac infection may result from non-coliform gram-negative contamination alone as a primary infection or in combination with other disease agents as complicating or secondary infection (Rudy, 1991; Sarma *et al.,* 1985). Infections may cause a respiratory disease from air sac infection; a septicemia disease from generalized infection; enteritis from intestinal infection; or a combination of any or all of these. In recent years, these infections have been recognized as a major cause of morbidity, mortality and condemnations in chickens. Infections which affect young birds may be resulted by the invasion of the microorganism through the unhealed navel or penetration from the egg shell prior to or during incubation (Haines and Moran, 1940; Smeltzer *et al.,* 1979). Proper egg handling, as well as a good hatchery management and sanitation program, are necessary to prevent early exposure. In Australia, morbidity of yolk sac infection in broilers was about 1–5% (Smeltzer *et al.,* 1979). In Iran, morbidity and mortality rates were 10% and 5–10% respectively (Bozorgmehri-fard, 1992). In addition, in Great Britain, mortality rate of yolk sac infection as a result of *Salmonella* was 2%.

Pseudomonas aeroginosa and *proteus volgaris* have pathogenicity for embryo or chick by protease enzyme (Harry, 1957). Several disinfectants can reduce egg shell contamination in hatching eggs.

In present study we evaluated the effect of formaldehyde on reducing non-coliform gram-negative bacteria contamination in hatching eggs.

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Materials and Methods

Two hundred and forty hatching eggs were selected from a broiler breeder farm and its hatchery, and sixty newly-hatched chicks were selected from the same hatchery based on a random design through different stages. We used formaldehyde gas for fumigation, using 40 c.c. of formalin and 20 grams of potassium permanganate crystals for each cubic meter of space. Fumigation was performed two times. Once, 2 hours after egg collection, and the second time, 24 hours later in hatchery. Stages include: Stage 1: Before cleaning and first disinfection, Stage 2: After first disinfection, Stage 3: Before setting inside the setter, Stage 4: Time of transferring from setter to hatcher, Stage 5: Newly-hatched chicks.

Each of egg shells and yolks and also yolk sacs of newly-hatched chicks were examined for existence of non-coliform gram-negative bacteria. At first egg shells were rinsed with peptone water solution inside sterile plastic bag and then these solutions were transferred to cysteine selenite media and were incubated in 37℃ for 24 hours, also these solutions were incubated separately in 37℃ for 24 hours. Then egg shells were sterilized by alcohol 70°, were broken, albumens were brought out, yolks were homogenized and were transferred to peptone water and cysteine selenite media and were incubated in 37℃ for 24 hours. Furthermore we sterilized abdominal skin of each of the newly-hatched chicks by alcohol 70°, then necropsied them, discharged their yolk sacs, homogenized and inoculated into peptone and cysteine selenite media and then incubated in 37℃ for 24 hours. After that we inoculated parts of colonies had grown on peptone water into McConky agar and blood agar and incubated them in 37℃ for 24–48 hours. Then we inoculated parts of colonies had grown on cysteine selenite media into Xlylose Lysine Deoxycholate (XLD) agar and incubated them in 37℃ for 24–48 hours. Afterward if colonies had not appeared, we incubated media for another24 hours and if colonies had appeared, we used Triple Sugar Iron (TSI) agar, Simon citrate, Methyl Red /Voges-Proskauer (MR/VP), motility, Indol and urease media to differentiate salmonella and E. Coli. Finally the results were analyzed by Chi-square test of SPSS software version 13.

Results

Results are summarized in Table 1 and Figure 1. In stage 1, from sixty egg shells, eleven egg shells had *Alcaligenes faecalis* contamination which is equall to 18.3% (Table 1). *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells in this stage. In stage 2, from sixty egg shells, seven egg shells had *Alcaligenes faecalis* contamination which is equall to 11.7% (Table 1). Again *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells like stage 1. In this stage contamination rate decreased by 6.6% in comparison to the previous stage (Fig. 1). In

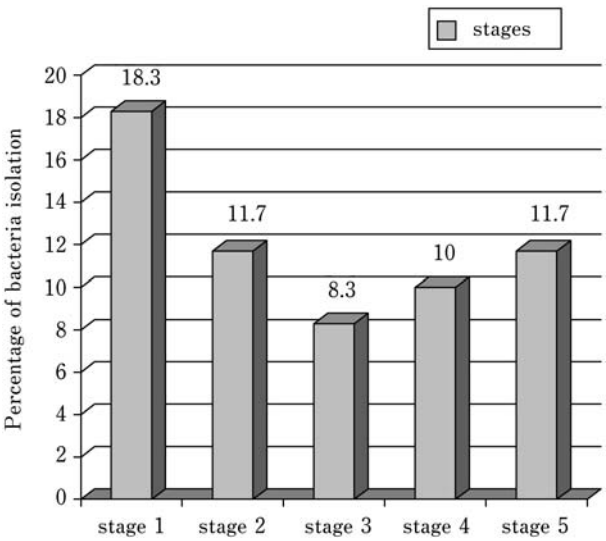

Fig. 1. **The frequency rates (%) of non-coliform gram-negative bacteria isolation at different stages.**
Stage 1: Before cleaning and first disinfection, Stage 2: After first disinfection, Stage 3: Before setting inside the setter, Stage 4: Time of transferring from setter to hatcher, Stage 5: Newly-hatched chicks.
Bar shows the percentage of non-coliform gram-negative bacteria isolation.

Table 1. **The frequency rates (%) of non-coliform gram-negative bacteria isolated from egg shells and yolk sacs at different stages**

‡No of Stages	No of Samples	No of Bacteria Isolated	*Frequency Rate
1	60	11◇	18.3%[a]
2	60	7◇	11.7%[ab]
3	60	5◇	8.3%[b]
4	60	6◇	10.0%[ab]
5	60	7◇	11.7%[ab]

* Different lower case superscript letters in a column indicate differences at $p < 0.05$.
◇ Alcaligenes faecalis was the only non-coliform gram-negative bacterium isolated.
‡ Stage 1: Before cleaning and first disinfection, Stage 2: After first disinfection, Stage 3: Before setting inside the setter, Stage 4: Time of transferring from setter to hatcher, Stage 5: Newly-hatched chicks.

stage 3, from sixty egg shells, five egg shells had *Alcaligenes faecalis* contamination which is equall to 8.3% (Table 1). Like previous stages, *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells. In this stage contamination rate decreased by 3.4% in comparison to the previous stage and decreased by 10.0% in comparison to the first stage (Fig. 1). In stage 4, from sixty egg shells, six egg shells had *Alcaligenes faecalis* contamination which is equall to 10.0% (Table 1). Similar to the past stages *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells. In this stage contamination rate increased by 1.7% in comparison to the previous stage (Fig. 1). In stage 5, from sixty yolk sacs, seven yolk sacs had *Alcaligenes faecalis* contamination which is equall to 11.7% (Table 1). Like previous stages *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated. In this stage contamination rate of yolk sacs was 11.7 (Fig. 1). Non-coliform gram-negative bacteria were not isolated from yolks in any stages.

Discussion

Although gram-positive bacteria are more common than gram-negative bacteria on egg shells immediately after eggs are laid, but gram-negative bacteria cause most infections which implies that they are capable of dominating the physical and chemical barriers of eggs. Egg shells have 7,000 to 17,000 pores through which surface bacteria can pass to the inside (Mayes and Takeballi, 1983).

Non-coliform gram-negative bacteria are causes of egg contamination and subsequent yolk sac infection. Surveys of meat-type flocks have reported the isolation of salmonellae from 94% of fecal samples in the Netherlands and 40% of breeder flock and broiler house sources in the United States (Byrd *et al.,* 1999). Cox *et al.* (1997) reported a decline in the incidence and level of *Salmonella* in commercial broiler hatcheries in the United States from 1990 to 1995. *Proteus* occasionally causes embryonic death, yolk sac infections, and mortality in young chickens.

Stage 1: *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells. Main source of egg contamination is the feces. Other sources of egg contamination include reproductive system of hen, dust, litter and hands of egg collector. Bacterial population on egg shell was mostly gram-positive because selected eggs were collected immediately after egg lay. On the other hand egg shell had high contamination because in this stage no cleaning or disinfection was done. Food pelleting is suggested as a useful procedure for reducing *Salmonella* contamination of broiler breeders (Bhatia and McNobb, 1980). Stresses can also shed *Salmonella* into the feces (Bhatia and McNobb, 1980). Barbour and Nabbut (1982) isolated *Salmonella* from rat and emphasized the role of rat in transmission of *Salmonella.* In addition, litter had 4% *Salmonella* contamination (Barbour and Nabbut, 1982). Egg shell contamination rate of *Salmonella* was 6% (Shareef *et al.,* 1997). In a research, gramnegative bacteria were isolated from 71% of 225 egg shells which included *Salmonella* and *Proteus* (Bastarows *et al.,* 1997). In another research, *Salmonella* was isolated from 14.3% of 2345 ceca of broiler breeders. *Salmonella* was isolated from egg shells of two chicken breeder farms in Saudi Arabia where in one farm contamination rate was 1.24% and in the other was 2.06% (Barbour and Nabbut, 1982). Humphrey *et al.* (1991) suggested that 0.6% of 5700 eggs in 15 farms had *Salmonella* contamination.

Stage 2: In this stage contamination rate decreased by 6.6% in comparison to the previous stage but was not significant ($p=0.273$). Again *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells. Eggs were cleaned by sand paper, graded and disinfected. Eggs would be cooled gradually after laying that causes contraction of the contents and therefore sucking microorganisms inside from pores. The reason of absence of bacteria in yolks is that egg collection, grading and disinfection were performed immediately after laying. Moats (1979) proved that using some disinfectants like quaternary ammonium compounds or sodium hypochlorite could decrease bacteria contamination rate but it was not very effective. Maris (1986) suggested that formaldehyde has most effect on reducing egg shell bacteria contamination and after that phenol and iodine compounds are effective. Knape *et al.* (2002) proved the role of cleaning and disinfecting of egg shell in reducing egg shell contamination. Smith *et al.* (2000) implied the role of surface humidity of eggs in the penetration of microorganisms.

So we conclude that *Alcaligenes faecalis* could not penetrate into the yolk if proper and rapid collection of eggs, utilization of suitable disinfectant and an optimum level of humidity were considered carefully while the high quality of shells accompanied this security process.

Stage 3: In this stage contamination rate decreased by 3.4% in comparison to the previous stage and decreased by 10.0% in comparison to the first stage. The reduction of contamination rate was not significantly different between this stage and stage 2 ($p=0.371$) but was significantly different compared with the first stage ($p=0.049$). Like previous stages *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated from egg shells. After transferring eggs from farm to hatchery, eggs were again disinfected by formaldehyde 24 hours after first disinfection. This procedure causes elimination of microorganisms from the eggs which were not disinfected properly at previous stage or were contaminated in transferring from farm to hatchery. Then eggs were stored in a cooling room until setting inside the setter. Although results showed that disinfection did not reduce contamination of egg shells significantly, it decreased by 10% compared with stage 1, which is the reason for prevention of non-coliform gram-negative bacteria multiplication.

Stage 4: In this stage contamination rate increased by 1.7% in comparison to the previous stage. Like previous stages *Alcaligenes faecalis* was the only non-coliform gram-

negative bacterium isolated from egg shells. Increase in contamination rate was not significant in comparison to the previous stage ($p=0.637$). Previous studies suggested that there is an increase in the number of egg shells bacteria between 19–21 days of incubation.

Stage 5: In this stage contamination rate of yolk sacs was 11.7%.Contamination rate increased by 1.7% in comparison to the previous stage. Like previous stages *Alcaligenes faecalis* was the only non-coliform gram-negative bacterium isolated. Increase in contamination rate was not significantly different ($p=0.670$) (Fig. 1). In this stage fluffs were the main cause of newly-hatched chicks contamination. Board (1966) and Cox *et al.* (1997) could not isolate any bacteria from albumen and yolk. Bozorgmehrifard (1992) did not isolate any bacteria from yolk either. Other researchers also had similar results (Mayes and Takeballi, 1983).Bhatia and McNobb (1980) and Chen (2000) suggested the role of fluffs in transmission. Another cause of yolk sacs contamination of newly-hatched chicks is meconium (Bhatia and McNobb). Entrance routes of microorganisms to newly-hatched chicks include unhealed navel, swallow or aspiration. Barbour and Nabbut (1982) isolated 19.23% *Salmonella* from day-old chicks. According to results non-coliform gram-negative bacteria were not isolated from yolks at stages 1–4, and the kind of contamination of newly-hatched chicks and egg shells was similar. So we can conclude that source of yolk sac contamination was the egg shell.

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