

Bacterial microflora of the chicken embryos and newly hatched chicken*

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ABSTRACT

The purpose of the study was to examine of bacteria present in chicken embryos and newly hatched chicks. The bacteria were determined quantitatively in caecal contents, liver and yolk sac of 25 embryos at 18 day of incubation, 25 embryos at 20 day incubation, and 25 newly hatched chicks. Gram-positive cocci belonging to the *Enterococcus* genus dominated in all samples examined and their population number amounted from 10^2 to 10^6 CFU/g in caecal contents, 10^2 - 10^4 CFU/g in yolk sac, and 10^3 CFU/g in liver. Other bacteria present in some samples were: *Staphylococcus* sp. 10^2 - 10^4 CFU/g, *E. coli* 10^6 CFU/g, *C. tertium* 10^5 CFU/g, *Klebsiella* sp. 10^4 CFU/g, and *Enterobacter* sp. 10^2 CFU/g. The results obtained indicate that the profile of gut microflora becomes more differentiated and their population number of particular groups higher with the age of embryos. Moreover, potentially pathogenic bacteria, including anaerobic *Clostridium tertium* may be isolated already during hatching period, suggesting the possibility of infection of embryos and newly hatched chicks.

KEY WORDS: chicken, embryos, hatching, gastrointestinal microflora

INTRODUCTION

Bacterial colonization of the alimentary tract in chickens begins usually during hatching process. The contact with bacteria from the environment, mainly through feed and water, allows the chick to develop intestinal flora with the first days after hatching. As soon as eggs are laid, bacteria penetrate the eggshell and other shell barriers (Berrang et al., 1999; Cook et al., 2003). Moreover, there is possibility

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of transovarian transmission for pathogenic bacteria, including *Salmonella* Enteritidis. Contamination of table eggs by *Salmonella* is essential for public health risk associated with consumption of infected eggs (Berrang et al., 1999). In contrast, contamination of hatching eggs may implicate invasion of these bacteria into egg contents and chicken embryos (Montgomery et al., 2005). There is limited number of reports in the literature indicating such route of infection of chicken embryos and newly hatched chickens (Tankson et al., 2002; Rosario et al., 2004). Furthermore, available results are based only on qualitative experiments.

The purpose of this study was quantitative examination of bacteria including anaerobes in caecal contents, livers and yolk sacs of broiler embryos and newly hatched chickens.

MATERIAL AND METHODS

Embryos and chickens

Twenty five broiler embryos at 18 day of incubation, 25 embryos at 20 day of incubation and 25 newly hatched chickens were obtained from a local hatchery. Only alive and completely developed embryos were selected for experiments. Caecum, liver and part of a yolk sac were taken aseptically for bacteriological examination.

Bacteriological examination

Direct microscopy smears were prepared from liver and mucosal scrapings from caecum followed by Gram staining. Caecal contents, livers and yolk sacs were collected aseptically and homogenized. Serial 10-fold dilutions were prepared in PBS. Volumes of 0.1 ml of each dilution were spread on freshly prepared agar media. Three replicate plates for each dilution were inoculated.

Following media and incubation conditions were used:

- blood agar (BioMérieux) for culture of aerobic bacteria (*Staphylococcus* sp. and *Micrococcus* sp.), incubated at 37°C for 24 h in aerobic conditions,
- Mac Conkey agar (BioMérieux) for culture of enteric Gram-negative bacteria, incubated at 37°C for 24 h in aerobic conditions,
- Kanamycin Esculin Azide Agar (Merck) for culture of enterococci, incubated at 37°C for 48 h in microaerophilic atmosphere generated by BBL™ CampyPak Plus system (Becton Dickinson),
- TSC (Merck) supplemented with D-cykloserine (0.4 mg/ml) for culture of *Clostridium* sp., incubated at 37°C for 48 to 72 h in anaerobic atmosphere generated by BBL™ GasPak system (Becton Dickinson).

Bacterial counts were calculated as colony forming units (CFU) per gram of sample established with the aid of plate count method. Different colony types were enumerated and isolated in pure culture. The identification of the isolated colonies was performed using standard bacteriological procedures described by Malicki and Binek (2004). Following criteria for identification were applied: colony character, cell morphology, Gram stain and API tests (BioMérieux).

RESULTS

The results from bacteriological examination of embryos and newly hatched chickens are shown in Table 1, Figures 1 and 5. Only single Gram-positive cocci were observed on smears prepared from caecal contents of 18 day embryos (Figure 1). There were no visible microorganisms on smears prepared from livers. *Enterococcus* sp. were found in caecal contents of 5 embryos, *Staphylococcus* sp. in 5 embryos and *Micrococcus* sp. in 2 embryos, with average number 1.9×10^2 CFU/g, 2.78×10^2 CFU/g and 1×10^2 CFU/g of caecal contents, respectively (Table 1). *Enterococcus* sp. (5 embryos) and *Staphylococcus* sp. (2 embryos) were found in yolk sac, with average number 1.06×10^2 CFU/g and 1.13×10^2 CFU/g of yolk sac contents. There was also single embryo, where *Micrococcus* sp. were found in yolk sac contents, reaching 1.5×10^2 CFU/g. The number of enterococci isolated from the liver of one embryo at 18 day of incubation reached 1×10^2 CFU/g of liver tissue.

Second group of embryos at 20 day of incubation have had more numerous microflora, composed mostly by Gram-positive cocci and in single cases by members of *Enterobacteriaceae* family. Direct smears prepared from caecal contents revealed numerous Gram-positive cocci, apart from one embryo, where spore-forming Gram-positive rods were also observed (Figure 2).

Enterococci were the most often isolated from caecal contents of 14 embryos at 20 days of incubation with average number 1.3×10^5 CFU/g. Additionally, there were also *Staphylococcus* sp. in 2 embryos with average number 3.66×10^4 CFU/g. *E. coli* and *Acinetobacter* sp. were isolated from caecal contents of single embryos, with number 1.6×10^3 CFU/g and 1.1×10^3 CFU/g. Unusually high level of *Enterococcus* sp. 5×10^5 CFU/g was detected in caecal contents of embryo, where we have observed spore-forming Gram-positive rods. However, the spore-forming rods, observed on direct smear were not isolated on TSC agar, because of extreme overgrowth of *Enterococcus* sp.

Bacteria isolated from yolk sacs of embryos at 20 day of incubation have been identified as *Enterococcus* sp. (10 embryos), *Staphylococcus* sp. (2 embryos), *Enterobacter* sp. (1 embryo) and their number reached 1.09×10^4 CFU/g, 2.72×10^3 CFU/g and 2.5×10^2 CFU/g, respectively. Following microorganisms have been found in livers of embryos: *Enterococcus* sp. (6 embryos) and *Enterobacter* sp. (1 embryo).

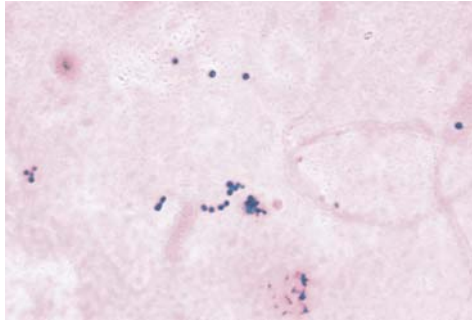


Figure 1. Direct smear prepared from caecal contents of chicken embryo (18 day of incubation), stained by Gram's method, magnification 1000 x

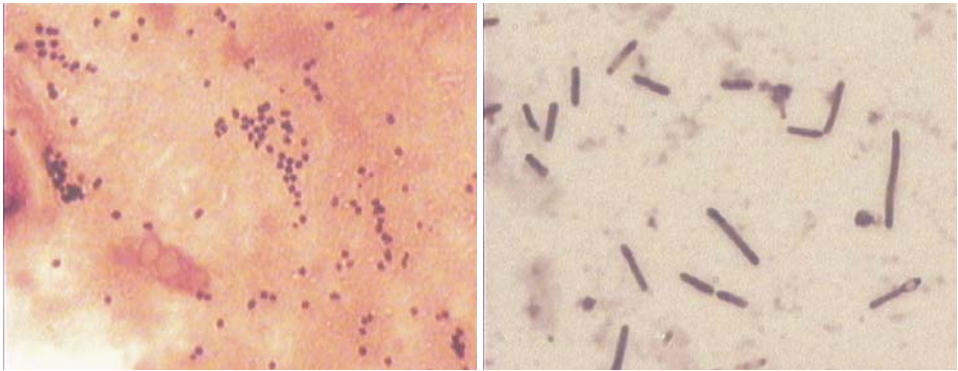


Figure 2. Direct smear prepared from caecal contents of chicken embryo (20 day of incubation), stained by Gram's method, magnification 1000 x

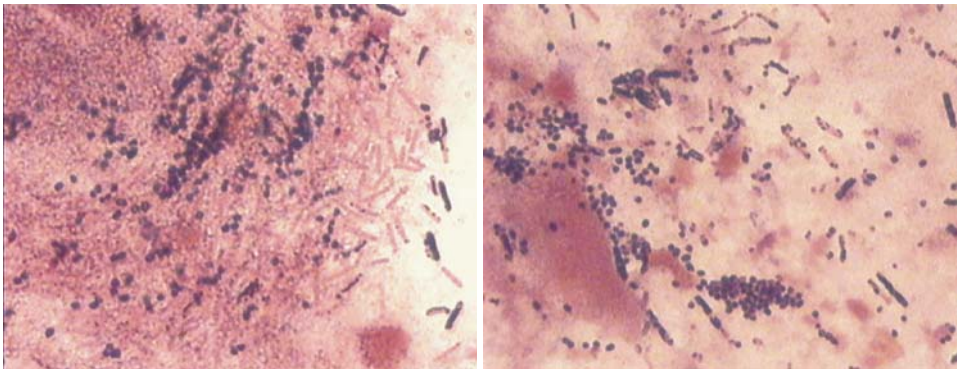


Figure 3. Direct smear prepared from caecal contents of newly hatched chicken stained by Gram's method, magnification 1000 x

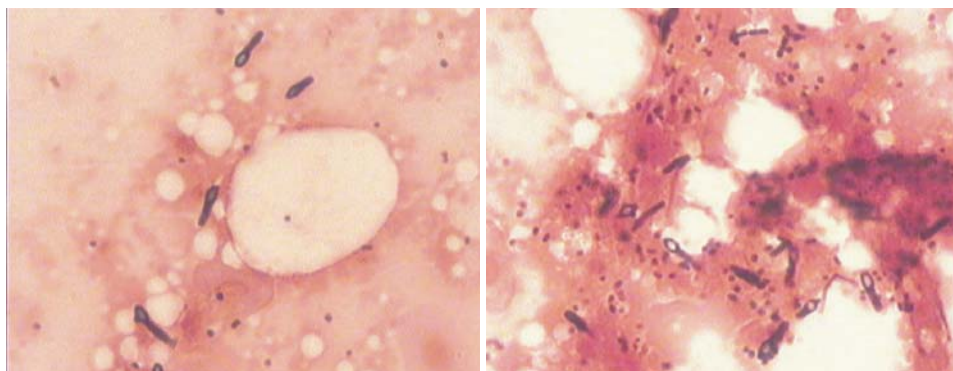


Figure 4. Direct smear prepared from livers of newly hatched chickens stained by Gram's method, magnification 1000 x

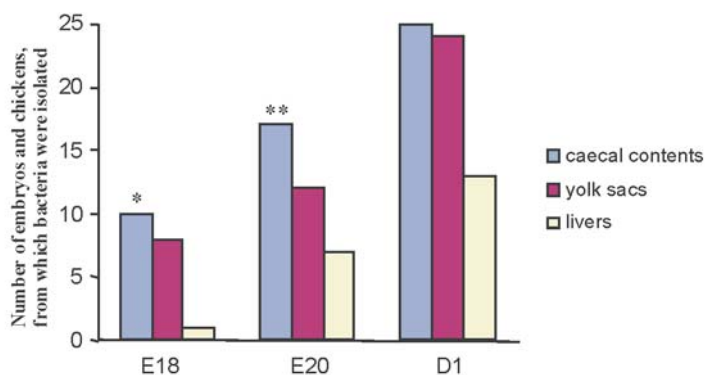


Figure 5. Number of chicken embryos and newly hatched chickens, from which bacteria were isolated from caecal contents, yolk sacs and livers. E18 - embryos at 18 day of incubation, E20 - embryos at 20 day of incubation, D1 - post hatching

* - the number of bacterial positive rate is lower as presented in Table 1, because bacteria belonging to three different groups were isolated from one embryo

** - the number of bacterial positive rate is lower as presented in Table 1, because bacteria belonging to two different groups were isolated from one embryo

The most complex microflora was isolated from chickens 3 h after hatching. Gram-positive cocci were isolated from caecal contents, livers and yolk sac (Table 1). Gram-positive cocci have been observed on direct smears prepared from caecal contents as well as from livers. In four cases numerous spore-forming Gram-positive rods were also present on smears from caecal contents (Figure 3) and livers (Figure 4).

Gram positive cocci belonging to *Enterococcus* sp. have been isolated from caecal contents each of 25 examined chickens, with average number 4.29×10^6 CFU/g. Additionally, *E. coli* were present in 14 chickens with average number

1.16×10^6 CFU/g. Also *Enterobacter* sp. (4 chickens) and *Klebsiella* sp. (2 chickens) were isolated from caecal contents.

The following bacteria were isolated from livers of young chickens: *Enterococcus* sp. (10 chickens) and *E. coli* (3 chickens). Bacterial microflora of yolk sack after hatching consisted of *Enterococcus* sp. (15 chickens), *E. coli* (6 chickens), *Staphylococcus* sp. (2 chickens) and *Enterobacter* sp. (1 chicken).

Bacteriological examination of four chickens, where spore-forming Gram-positive rods were observed, allowed obtaining on TSC agar supplemented with D-cykloserine anaerobic rods, with average number 1.01×10^5 CFU/g of caecal contents. All isolated strains were identified with API 20A as *Clostridium tertium* (%id=98.9).

DISSCUSION

In transovarial infection bacteria are transmitted from the ovary or other part of the reproductive tract to the yolk. Moreover, penetration of pathogenic bacteria to the egg contents may appear during incubation period, leading to infection of embryo. Yolk sack infection (YSI) is economically important disease since it increases young chicken's mortality ratio during first week of life. Birds that survive YSI show poor weight gain as well as poor carcass quality. Factors affecting bacterial penetration through the egg shell include : faecal contamination of shells, lack of hygiene at the farm, incubation of eggs with eggshell defects, collection of eggs laid on the floor or inappropriate egg disinfection (Rosario et al., 2004).

Several reports were found in the literature evaluating bacterial microflora of chicken eggshells. It was reported by Cook et al. (2003), that *Staphylococcus* sp., *Enterococcus* sp. and *Micrococcus* sp. are predominate bacteria isolated from eggshell. However, these microorganisms, except *S. aureus* and *E. faecalis* do not appear to be pathogenic for embryos and newly hatched chickens. Additionally, above mentioned authors showed that Gram-positive rods, Gram-positive cocci and *Enterobacteriaceae* are the major contaminants on the eggshell. By contrast, microbiota of egg albumen and yolk consist mostly of Gram-positive cocci, indicating their ability to invade through the eggshell.

In contrast, there is a limited number of publications covering bacterial microflora of chicken embryos and newly hatched chickens. In general, our results are in line with these uncommon reports. Results presented in this study provide quantitative analysis of bacterial counts from caecal contents, livers and yolk sacs, including anaerobic microorganisms. In our findings Gram-positive cocci were present in some embryos at 18 and 20 day of incubation; however *Enterococcus*

sp. were most common. *Enterobacteriaceae* were isolated only in single cases. The number of bacteria were higher in embryos one day before hatching as compared with those on 18 day of incubation. Chickens were proved to have the most complex microflora, including significant *E. coli* presence. Binek et al. (2000) reported isolation of *Enterococcus* sp., *Micrococcus* sp. and *Bacillus* sp. from colon of chicken embryos at 17, 18 and 19 day of incubation. Similarly, Tankson et al. (2002) determined the bacterial microflora in heart and lungs of embryos and young chickens and proved that bacteria could be isolated from healthy chickens starting from 17 day of incubation, enterococci were isolated the most frequently.

In the present study *Enterococcus* sp. were the most often isolated from embryos and chickens. *Enterococcus faecalis* is opportunistic, Gram-positive, facultative anaerobe, found in the intestinal tract of humans and many animals including chicken. It is suggested that *E. faecalis* is as one of the etiological agents of pulmonary hypertension syndrome (PHS) in broiler chickens, which have become a serious problem in fast growing broilers recently (Tankson et al., 2001). PHS occurs when blood pressure in the pulmonary tree increases. Back pressure causes that right ventricle of the heart become overworked. As a result it becomes hypertrophic and abdominal cavity is full of fluid. It is well proved, that challenge with *E. faecalis* caused pulmonary hypertension, however the role of *E. faecalis* in PHS is not fully understood. It should be taken under consideration, that embryos and chickens, from which *Enterococcus* sp. were isolated, are at high risk of PHS.

Ultimately, the most interesting point of our findings was the isolation of anaerobic *Clostridium tertium* from newly hatched chickens connected with significant numbers of *Enterococcus* sp. in examined organs. *Clostridium tertium* were obtained from 4 newly hatched chickens. It suggested that spore forming bacteria are able to resist disinfection process and contaminate egg shells. During incubation they can penetrate the egg and infect embryo.

C. tertium is non-toxin-producing, aerotolerant microorganism, which is usually considered as nonpathogenic (Steyaert et al., 1999; Miller et al., 2001). However, there are reports of this species causing bacteraemia, concerning in most cases neutropenic people. Silvera et al. (2003) proved its ability to produce mild enteritis in cattle in experimental conditions. *C. tertium* has been also reported as a cause of megacolon and chronic diarrhoea in cockatoo (Hess et al., 1998). Based on the analysis of the results reported here, it can be concluded, that *C. tertium* appears to be an emerging pathogen in veterinary. Furthermore, its ability to produce spores, aerotolerant nature, ability to cause diarrhoea in animals and presence in gastrointestinal tract of healthy people and animals, make it a possible food-contaminating microorganisms. Additional experiments would be

necessary to determine the prevalence of *C. tertium* infections in embryos and chickens.

CONCLUSIONS

In conclusion, it is reasonable to assume that bacteria, including spore-forming *C. tertium* could be isolated from gastrointestinal tract of chicken embryos and newly hatched chickens. These results tend to support the possibility that *C. tertium* could contaminate eggshells and infect embryos and chickens. If this assumption is correct, appropriate preventive countermeasures could be implemented in the poultry industry.

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