Prevention of *Salmonella* Colonization in Neonatal Broiler Chicks by Using Different Routes of Probiotic Administration in Hatchery Evaluated by Culture and PCR Techniques

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**ABSTRACT**

The effects of various methods of probiotic administration in hatchery and on prevention of *Salmonella enteritidis* (*Se*) in broiler chicks was investigated. A total of 150 *Salmonella* free day old chicks (Ross 308) were assigned to five experimental groups including control and four in-hatchery probiotic administration method groups comprised of: *in ovo* injection, oral gavage, spray and vent lip application. Each group was comprised of 30 chicks. The chicks were challenged by 8 Log CFU *Se* using oral gavage on 2 days of age. At 1 and 7 days of post-challenge (PC) 15 birds per experimental group were sampled for *Se* recovery through either one of culture or culture based PCR techniques. Administration of probiotics reduced the number of *Se* colonized chicks, compared with control as evaluated through either culture or PCR method. These reductions were significant for all the administration routes (*P* < 0.05), except for the 1 day PC, evaluated by culture method (*P* > 0.05). Furthermore probiotics were capable of reducing the number of colonized chicks from day 1 to day 7 PC. Vent lip method was evaluated as the most effective route of probiotic administration in prevention of *Se* colonization, not significantly different from either spray application in day 1 of PC group or from other administration methods in the day of 7 PC (*P* > 0.05). PCR method was more responsive in detection of *Se* as compared to traditional culture method. Administration of probiotics in hatchery finally resulted in reducing the colonization of *Salmonella* in the alimentary tract of chicks.

**Keywords**: Administration method, Broiler, Hatchery, Probiotic.

**INTRODUCTION**

Modern practices in the poultry industry include artificial incubation. With such management procedures the colonization of the enteric tract of newly hatched chicks by desirable microorganisms is delayed as compared to chicks hatched in contact with their adult birds. Therefore, alimentary tract can be easily colonized by pathogenic bacteria (Flower and Mead, 1989). Under natural conditions, microorganisms that are initially established usually remain for the rest of life in the alimentary tract of the birds.

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Therefore, gut colonization in the beginning of life could promote a natural barrier impeding the colonization and multiplication of Salmonella and other pathogenic bacteria in the alimentary tract (Flower and Mead, 1989; Olivera et al., 2000; Ghadban, 2002). The risk of Salmonella infection in young birds is still high even if a competitive exclusion product is administered. Some serotypes, like Se can be transmitted vertically (Gast, 1997; Berchieri, 2000) and spread rapidly among young birds (Oliveria et al., 2000). Despite having contact with birds infected with Salmonella, other birds might be protected by competitive exclusion (CE) techniques. According to Oliveira et al. (2000), colonization of the intestinal tract is fast in using CE techniques and helps prevent infection. In addition, there are indications that the desired effect can be achieved even after infection, as reported by Ziprin et al. (1993). Thus, the concept of Nurmi has been recommended worldwide as part of Salmonella control programs in birds; although it is still not clear how long it takes to effectively protect the birds. Application to large numbers of chicks under commercial conditions must be efficient, should be administered as early in life as possible (Schnetiz et al., 1992) and should minimize the effects of such uncontrolled variables as water quality and porportioner/medicator function and consistency (Wolfenden et al., 2007).

On the other hand, the efficiency of probiotics depends on, strain and dosage of probiotic, age of bird and as well on the route of administration. Different methods of probiotic administration in hatchery were described as: in ovo injection (Cox et al., 1992; Edens et al., 1997), spray administration (Pivinick and Nurmi, 1982; Wolfenden et al., 2007), oral gavage (Sterzo et al., 2005; Higgins et al., 2007), and vent lip (Filho et al., 2007; Higgins et al., 2008). There is a lack of a study that compares all these administration routes. In this study, various methods of probiotic administration to prevent Salmonella colonization in neonatal broiler chicks was evaluated. Furthermore the efficiency of PCR procedure with pre-enrichment was compared with the standard culture of Salmonella that is routinely used in the poultry industry, as diagnostic and quantitative tools, for evaluating the Salmonella contamination.

MATERIALS AND METHODS

Salmonella

Se (RITCC1695) was originally purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran). For preparation of the inocula, bacteria were grown in nutrient broth (Merck, Germany) at 37°C for 24 hours. The viable cell concentration of the inoculums was determined by counting the colony forming units (cfu) on XLD-agar (Merck, Germany) plates, following a pour plate procedure (Bjerrum et al., 2003).

Experimental Chicks

The study was carried out according to guide to the care and use of experimental animals (Tarbiat Modares University, College of Medical Sciences). One hundred and fifty day old broiler (Ross 308) Salmonella free chicks were assigned to one of each four groups of different methods of probiotic administration and as well to a control group. Each group, comprised of 30 chicks was kept in a cage battery(90×60×40 cm³) and fed ad libitum during the 10 days of the experiment. To ensure freedom from Salmonella contamination, the feed was analyzed before the experiment, following an enrichment procedure (Barrow and Tucker, 1986).

Probiotic Administration Groups and Challenge

Protexin Concentrate® (Probiotics International, UK., consisting of 9
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A microorganism with a total count of up to $2 \times 10^9$ cfu g$^{-1}$ of: Aspergillus oryzae, Lactobacillus acidophilus, L. rhamnosus, L. plantarum, L. bulgaricus, Bifidobacterium bifidum, Enterococcus faecium, Streptococcus thermophilus and Candida pinitolopesii was used as probiotic preparation due to its capability of forming a consistent suspension in water. The four administration method groups were: (1) In ovo injection group; after 18 days of incubation, 40 fertile eggs were injected (into their air cells) with 0.1 ml of probiotic containing $7 \times 10^7$ cfu ml$^{-1}$ per egg in sterile PBS. (2) Oral gavage group; 0.1 ml of probiotic suspension containing $7 \times 10^7$ cfu ml$^{-1}$ administrated through gavage into the crop. (3) Spray administration; conducted by confining chicks in their shipping box and being directly sprayed with 0.25 ml of probiotic suspension containing $7 \times 10^7$ cfu ml$^{-1}$ per each chick. The chicks were then held in their shipping box for 30 minutes before being placed in the cage. (4) Vent lip group; this method is based on the phenomenon known as cloacal drinking (Sorvari et al., 1975); each chick received 25 µl of probiotic suspension containing $2.8 \times 10^8$ cfu ml$^{-1}$ deposited on the vent lip. The drop was sucked inside the cloaca within a few seconds. (5) Control group, chicks did not receive any probiotic treatment. One day after the placement all chicks were individually challenged orally (0.1 ml) with Se at approximately $10^8$ cfu chick$^{-1}$.

**Se Recovery**

For the recovery test of Se, chicks were humanly killed by CO$_2$ asphyxiation at 1 and 7 days PC (n=15 birds per group). One gram of cecal contents was aseptically removed and placed into sterile tubes containing 9 ml of peptone water buffer and incubated overnight at 37°C. To be enriched in a selective media 0.1 ml of each tube was transferred to 10 ml of Rappaport Vassiliadis (RV) broth (Merck, Germany) and incubated overnight at 37°C.

Following enrichment, each sample of cecal contents was streaked for isolation on XLD agar plates. The plates were incubated at 37°C for 24 hours and then observed for either the presence or absence of characteristic Salmonella colonies, (black on XLD plate). The identity of assumed Se colonies was further confirmed by culture on TSI and urea agars (Merck, Germany). The recovery of Se is reported as the number of positive samples/total number of samples. Furthermore 2 ml of cultured RV broth was taken for subsequent DNA extraction.

Cecal content samples from each bird was serially diluted and spread-plated on XLD agar, incubated for 24 hours at 37°C and the CFU of Se per gram of cecal content determined. Salmonella colony counts were expressed as Log 10 per gram of cecal content.

**DNA Extraction**

Cells were harvested from 1 ml of RV broth by centrifugation at 8000g for 5 min. DNA extraction was carried out according to Hai-Rong and Ning (2006); else RNA digestion step was omitted.

**PCR**

The primers used in study are shown in Table 1. Universal primer identifies all known bacteria using invariant region in the 16s rDNA of the bacteria. The universal primer set was used for determining the total bacterial population. Primers targeting Salmonella species were from the 201 bp region and from the 597 bp region of rDNA sequence (Gene Bank accession # AF332600). For PCR amplification of the bacteria, 5 µl of DNA extract was added to 45 µl of the PCR mixture containing 30.875 µl of nuclease free water, 2 µl of each primer (10 µM), 2 µl dNTP mixes (10mM), 5 µl PCR buffer, and 0.0625 µl Taq...
polymerase. PCR components were provided by Cinagen, Iran. The PCR was conducted in programmable thermal controller (BioRad, USA). The amplification conditions were: 1 cycle of 94°C for 4 minutes, 35 cycle of 94°C for 30s, 60°C for 1 minute and 68°C for 1.5 minutes, and finally 1 cycle of 68°C for 1.5 minutes (Amit-Romach et al., 2004). PCR products were visualized by agarose gel (1%) electrophoresis containing ethidium bromide (Serva, Germany). Densitometric evaluation of different bands was carried out using Photo Capt software version 12.4 (Vilber Lourmat, France). The densitometry results were reported as relative density of Salmonella bands to universal bands.

### Statistical Analysis

The Se enumeration and band density ratio data were analyzed in completely randomized design model, the comparison of means being carried out through LSD test. The number (%) of Se colonized chicks data were analyzed using Chi Square Test. Significance level was considered at P< 0.05. All statistical analyses were done using SAS program (SAS Institute, 1998).

### RESULTS

#### Se Counts in Cecal Contents

The probiotic administration in general reduced Salmonella contamination (Log CFU g⁻¹) in cecal content of samples taken at 1 and 7 days of PC (Table 2). In ovo injection, spray and vent lip administration of probiotic significantly reduced contamination at day 1 of PC (P< 0.01), with the latter being the most effective route (2.4 Log CFU g⁻¹ reduction as compared to control). At day 7 PC, oral gavage, spray and vent lip significantly reduced the Salmonella counts in cecal contents of colonized chicks (P< 0.05). Se counts increased in cecal contents of colonized chicks from day 1 to day 7 PC. As evaluated by PCR method (Table 3) probiotic administration reduced the relative content of Salmonella to total cecal bacterial population represented by ratio of Salmonella band to universal band (P< 0.01). The amplified products for both Salmonella and universal primers are depicted in Figure 1. Based upon these results, it seems that Salmonella

### Table 1. PCR primers employed in the study a.

<table>
<thead>
<tr>
<th>Bacterial group</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal</td>
<td>Forward</td>
<td>CGTGCCAGCGCCGTAATACG</td>
<td>611</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGTTGCGCTCGTTGGGACTTAACCCAACAT</td>
<td>396</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Forward</td>
<td>CGGGCCTCTTGCCATCAGGTG</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACATCCGACTTGACAGACCG</td>
<td>152</td>
</tr>
</tbody>
</table>

a (Amit-Romach et al., 2004).

### Table 2. Effect of in-hatchery probiotic administration methods on Se counts in cecal contents of colonized chicks at d 1 and 7 post-challenge evaluated by culture method (Log cfu g⁻¹).

<table>
<thead>
<tr>
<th>Administration method</th>
<th>Log CFU g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.2 ± 0.9 a</td>
</tr>
<tr>
<td>In ovo injection</td>
<td>4.9 ± 0.9 bc</td>
</tr>
<tr>
<td>Oral gavage</td>
<td>5.5 ± 1.0 ab</td>
</tr>
<tr>
<td>Spray</td>
<td>4.9 ± 1.2 bc</td>
</tr>
<tr>
<td>Vent lip</td>
<td>3.8 ± 0.9 c</td>
</tr>
</tbody>
</table>

* Significant difference (P< 0.05); ** Significant difference (P< 0.01); Values are Mean±SEM.
anyhow, the number of Se colonized chicks was reduced from day 1 to day 7 PC as evaluated by culture and through PCR methods (Table 4).

Comparison of Se Recovery by Culture and PCR Method

The results of conventional culture method vs. PCR method are presented in Table 4. All administration routes reduced the number of cecal-culture-Se positive chicks, when screened using the PCR method. There were no effects observed at 1 day PC when screened using the culture method. Reduction in number of Se infected chicks due to probiotic administration by contamination decreased from day 1 to 7. Anyhow, the number of Se colonized chicks was reduced from day 1 to day 7 PC as evaluated by culture and through PCR methods (Table 4).

**DISCUSSION**

Based upon the results of this experiment, administration of probiotics in hatchery significantly reduced Salmonella recovery from cecal contents of neonatal broiler chicks as compared with untreated control at day 1 and at day 7 PC. Mead (2000) proposed 4 methods by which competitive exclusion cultures are able to exclude enteric pathogens: competition for receptor sites, production of bacteriocins, production of volatile fatty acids that are inhibitory of certain enteric pathogens or competition with pathogens and native flora for limiting day 1 (P< 0.05) and by day 7-PC (P< 0.01) was evidenced as by both (culture and PCR) methods.

Table 4. Effect of in-hatchery probiotic administration methods on Se recovered from cecal contents at d 1 and 7 post-challenge evaluated by PCR method a.

<table>
<thead>
<tr>
<th>Administration methods</th>
<th>Day post-challenge</th>
<th>1 **</th>
<th>7**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.39±0.24 a</td>
<td>0.14±0.15 c</td>
</tr>
<tr>
<td>In ovo injection</td>
<td></td>
<td>0.2±0.24 b</td>
<td>0.04±0.04 c</td>
</tr>
<tr>
<td>Oral gavage</td>
<td></td>
<td>0.32±0.3 ab</td>
<td>0.14±0.15 c</td>
</tr>
<tr>
<td>Spray</td>
<td></td>
<td>0.23±0.24 b</td>
<td>0.12±0.07 c</td>
</tr>
<tr>
<td>Vent lip</td>
<td></td>
<td>0.15±0.24 b</td>
<td>0.14±0.07 c</td>
</tr>
</tbody>
</table>

* Ratio of Salmonella band density to universal band density. ** Significant difference (P<0.01)

Table 3. Effect of in hatchery probiotic administration methods on Se proportion in cecal contents at d 1 and 7 post-challenge evaluated by PCR method a.

<table>
<thead>
<tr>
<th>Number of chicks colonized /Number of chicks challenged (%)</th>
<th>Administration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture *</td>
</tr>
<tr>
<td>Control</td>
<td>12/15 (80)</td>
</tr>
<tr>
<td>In ovo injection</td>
<td>9/15 (60)</td>
</tr>
<tr>
<td>Oral gavage</td>
<td>10/15 (64)</td>
</tr>
<tr>
<td>Spray</td>
<td>8/15 (53)</td>
</tr>
<tr>
<td>Vent lip</td>
<td>5/15 (33)</td>
</tr>
</tbody>
</table>

* and **: Significant at the 0.05 and 0.01 levels, respectively.

ns: Non significant.
Based on the present results (Table 4) using PCR technique could detect more infected chicks as compared with traditional culture method, thus amplification of DNA sequences, unique to an organism by PCR, improves the speed and sensitivity at which organisms can be detected. This is in agreement with Annamária et al. (2006) and Bailey (1998).

The number of Se colonized chicks decreased from day 1 to day 7 PC (Table 4), although Se counts in cecal content of Se colonized chicks, when evaluated by culture method, were raised from day 1 to day 7 PC (Table 2), while the ratios of Salmonella to other bacteria in cecal contents, as evaluated by PCR, were decreased (Table 3). This observed contradictory could be explained by the fact that while Se was increasing during PC days in colonized birds, it was outpaced by developing microflora in the alimentary tract which can hamper the relative presence of Se in cecal contents. This should be addressed by the rational differences between the two methods of evaluation used in this study, namely: culture method which determines the absolute Se population versus PCR method in which the relative presence of Se in microflora is determined. In this experiment, administration routes which deliver the whole dose of probiotic directly into alimentary canal (oral gavage and vent lip) were the most effective administration routes, which seemed to be the result of direct delivery of the whole dose of probiotic microorganisms into the target sites. Se colonized chicks carried reduced Se numbers when the probiotic was delivered by vent lip as compared to oral gavage, presumably due to more direct access to the lower small intestine and cecum, bypassing the more hostile action of low gastric pH and upper small intestine enzymatic as well as bile actions (Cox et al., 1990).

In conclusion, administration of probiotics in hatchery is effective in protecting the chicks’ digestive tract against Se colonization. Vent lip application of probiotic was evaluated as the most effective method of probiotic administration; however from a practical point of view this method is far from routine in hatchery practices. The spray application of probiotics produced results not significantly different from those in vent lip route, thus, spray method could offer a low-cost and an efficient tool in commercial application of probiotics.

ACKNOWLEDGEMENTS

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ارزیابی تأثیر تجویز پروپتیک در جوجه کشی بر پیشگیری از سالمونلا با استفاده از روش‌های کشت و واکنش زنجیری پلیمراز

ز. هاشم زاده، م. ا. کریمی ترشی، ش. رحیمی، و. رزبان و ت. زهراپی صالحی

چکیده

در این بررسی تأثیر روشهای مختلف تجویز پروپتیک در جوجه کشی بر پیشگیری از عفونت سالمونلا، انتخاب‌های مورد ارزیابی قرار گرفت. 15 قالب جوجه غوشته سویه‌وراس (308) به پنج گروه شماری گروه شاهد و چهار گروه روشهای مختلف تجویز پروپتیک در جوجه کشی شامل تزریق به تمام مرغ، فاشن‌ها، گاواز دهانی و تلقیح در کل‌ها به ترتیب به ترتیب شد. هر گروه شامل 30 قالب جوجه Log CFU بود. همه پرندگان یک روز بعد از دریافت پروپتیک با PCR گاواز دهانی چالش داده شدند. یک و هفت روز بعد از چالش برای پزشکی سالمونلا اختصاص داده شدند. PCR و کشت 15 پرندگان هر گروه نمونه‌برداری شدند. در مقایسه با گروه شاهد، تجویز پروپتیک تعداد پرندگان استفاده شده در گروه‌های آموزشی کاهش داشت که از تکنیک برای شناسایی سالمونلا استفاده کردیم (P<0.05). اما اختلاف معنی‌داری زمان استفاده از تکنیک PCR کشته یک روز پس از چالش مشاهده نشد (P>0.05). تجویز پروپتیک تعداد پرندگان عفونی را از یک روزگاه تا هفت روزگاه کاهش داد. این مطالعه نشان داد که موتورترین روش تجویز پروپتیک در پیشگیری از سالمونلا روش تلقیح به کلواک می‌باشد که تفاوت معنی‌داری با گروه آفتابنی در یک روز بعد از چالش با یکی از گروه‌های دارنده (P<0.05). روش PCR سبب یافته بیشتری برای شناسایی سالمونلا در مقایسه با روش کشت شناسایی داد. تجویز پروپتیک در جوجه کشی قادر به کاهش سالمونلا انتخاب‌های محصول خوش‌گویی گوشته بود.