J. Agr. Sci. Tech. (2010) Vol. 12: 425-432

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 comprized 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 reponsive 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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[DOR: 20.1001.1.16807073.2010.12.4.7.9]

(Savage, 1987). 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 cm^3) 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

[DOR: 20.1001.1.16807073.2010.12.4.7.9]

microorganisms with a total count of upto 2×10^9 cfu g^{-1} of: *Aspergillus oryzae, Lactobacillus acidophilus, L. rhamnosus, L. plantarum, L. bulgaricus, Bifidobacterium bifidum, Enterococcus faecium, Streptococcus thermophilus* and *Candida pintolopesii*) 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 7×10^7 cfu ml^{-1} per egg in sterile PBS. (2) Oral gavage group; 0.1 ml of probiotic suspension containing 7×10^7 cfu 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 7×10^7 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 2.8×10^8 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*

[DOR: 20.1001.1.16807073.2010.12.4.7.9]

Table 1. PCR primers employed in the study [a].

Bacterial group	Primer	Sequence (5´-3´)	Length (bp)
Universal	Forward	CGTGCCAGCCGCGGTAATACG	611
	Reverse	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT	
Salmonella	Forward	CGGGCCTCTTGCCATCAGGTG	396
	Reverse	CACATCCGACTTGACAGACCG	

[a] (Amit-Romach *et al*., 2004).

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 a 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 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}).

	Time post-challenge (d)	
	1**	7*
Administration method	Log	CFU g^{-1}
Control	6.2 ± 0.9 [a]	10.51 ± 1.29 [a]
In ovo injection	4.9 ± 0.9 [bc]	8.75 ± 1.14 [ab]
Oral gavage	5.5 ± 1.0 [ab]	7.93 ± 1.95 [b]
Spray	4.9 ± 1.2 [bc]	8.28 ± 0.98 [b]
Vent lip	3.8 ± 0.9 [c]	7.45 ± 2.76 [b]

* Significant difference (P< 0.05); ** Significant difference (P< 0.01), [1]Values are Mean+SEM.

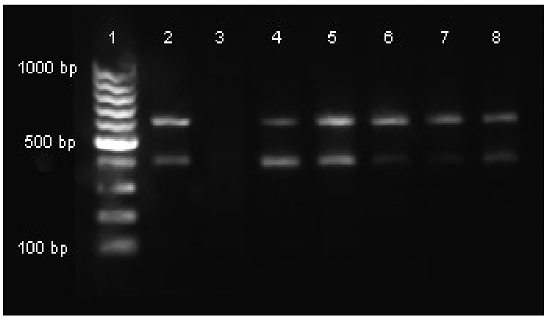

Figure 1. Agarose gel electrophoresis of PCR products from cecal contents of different individuals on experimental treatments at d 7 PC (lanes 4-8, control, *in ovo* injection, oral gavage, spray, and vent lip administration of probiotics, respectively). Lane 1, 100 bP DNA size marker, lane 2, positive control, lane 3, negative control. *Salmonella* band (396 bp) and universal band (611 bp).

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].

Administration methods	Day post-challenge	
	1 **	7**
Control	0.39±0.24 [a]	0.41±0.25 [a]
In ovo injection	0.2±0.24 [b]	0.22±0.22 [b]
Oral gavage	0.32±0.3 [ab]	0.12±0.07 [c]
Spray	0.23±0.24 [b]	0.14±0.15 [c]
Vent lip	0.15±0.24 [b]	0.04±0.04 [c]

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

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).

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 day 1 (P< 0.05) and by day 7-PC (P< 0.01) was evidenced as by both (culture and PCR) methods.

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

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

	Number of chicks colonized /Number of chicks challenged (%)			
	1(d)		7(d)	
Administration method	Culture ns	PCR*	Culture**	PCR**
Control	12/15 (80)	15/15 (100)	12/15 (80)	15/15 (100)
In ovo injection	9/15 (60)	11/15 (73)	8/15 (53)	10/15 (66)
Oral gavage	10/15 (64)	11/15(73)	3/15 (20)	5/15 (33)
Spray	9/15 (60)	11/15 (73)	4/15 (27)	5/15 (26)
Vent lip	7/15 (46)	7/15 (46)	2/15 (13)	4/15 (26)

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

[DOR: 20.1001.1.16807073.2010.12.4.7.9]

nutrients.

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 offere a low-cost and an efficient tool in commercial application of probiotics.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the Research Council of Tarbiat Modares University for the financial assistance. They would also like to thank Mr. John Tuer for reviewing the manuscript and his other positive contributions.

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[DOR: 20.1001.1.16807073.2010.12.4.7.9]

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

ز. هاشم زاده، م. ا. کریمی ترشیزی، ش. رحیمی، و. رزبان و ت. زهرایی صالحی

چکیده

در این بررسی تاثیر روشهای مختلف تجویز پروبیوتیک در جوجه کشی بر پیشگیری از عفونت سالمونلا انتریتیدیس در جوجه‌های گوشتی مورد ارزیابی قرار گرفت. ۱۵۰ قطعه جوجه گوشتی سویه راس (۳۰۸) به پنج گروه شامل گروه شاهد و چهار گروه روشهای مختلف تجویز پروبیوتیک در جوجه کشی شامل تزریق به تخم مرغ، افشانه، گاواژ دهانی و تلقیح در کلواک تقسیم شدند. هر گروه شامل ۳۰ قطعه جوجه بود. همه پرندگان یک روز بعد از دریافت پروبیوتیک باLog CFU ۸ سالمونلا انتریتیدیس از طریق گاواژ دهانی چالش داده شدند. یک و هفت روز بعد از چالش برای بازیابی سالمونلا انتریتیدیس از محتویات سکوم و لوزه سکومی با دو تکنیک PCR و کشت، ۱۵ پرنده از هر گروه نمونه‌برداری شدند. در مقایسه با گروه شاهد، تجویز پروبیوتیک تعداد پرنده‌های عفونی را کاهش داد زمانی که از تکنیک PCR برای شناسایی سالمونلا استفاده کردیم (P<۰/۰۵). اما اختلاف معنی‌داری زمان استفاده از تکنیک کشت یک روز پس از چالش مشاهده نشد (P>۰/۰۵). تجویز پروبیوتیک تعداد پرنده‌های عفونی را از یک روزگی تا هفت روزگی کاهش داد. این مطالعه نشان داد که موثرترین روش تجویز پروبیوتیک در پیشگیری از سالمونلا روش تلقیح به کلواک می باشد که تفاوت معنی داری با گروه افشانه در یک روز بعد از چالش و با سایرگروه‌ها در هفت روز بعد از چالش ندارد (P<۰/۰۵). روش PCR حساسیت بیشتری را برای شناسایی سالمونلا در مقایسه با روش کشت نشان داد. تجویز پروبیوتیک در جوجه کشی قادر به کاهش سالمونلا انتریتیدیس در دستگاه گوارش جوجه‌های گوشتی بود.

[DOR: 20.1001.1.16807073.2010.12.4.7.9]