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Competitive exclusion against *Salmonella enterica* subspecies *enterica* serovar Enteritidis infection in chickens

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ABSTRACT

A total of 250 newly-hatched broiler chickens allocated to two groups were used and two replicate trials were carried out. The chicks were dosed by gavage with commercial competitive exclusion (CE) product (trial groups) or with 0.5 ml of physiological saline (controls) at day 0, day 1 and day 5. On day 2, eight birds per tier were challenged by oral gavage with 0.5 ml of an approximate 10^7 organism/ml dilution of *S.* Enteritidis phage type 9a and were used as seeders. On the fifth day after challenge, and at 14 and 21 days of age, 12 chicks from each group were selected and Salmonellae were counted in their caeca. On days 14 and 21 the proportion of infected animals was significantly lower ($P< 0.05$) in the group treated with CE product than in the challenge controls. One-half of the treated animals remained infected on day 14, and only one-quarter one week later. In the control group the proportion of infected animals reached 21/24 and 17/24 on days 14 and 21, respectively. There was no significant difference ($P>0.1$) among treated groups with regard to the spleen colonization observed on days 14 and 21. On day 14, the number of chickens with infected spleen was 4/24 in challenge control and 9/24 in the treated group. On day 21 this proportion was 4/24 and 3/24, respectively. Commercial competitive exclusion product provided a significant protective effect against pathogenic *S.* Enteritidis PT 9a used for challenge.

Key words: *Salmonella* Enteritidis, competitive exclusion, chicken, salmonellosis

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Introduction

Salmonellosis is an important public health problem worldwide. Many incidents of this disease have been reported to be caused by *Salmonella enterica*, subspecies *enterica* serovar Enteritidis *(S.* Enteritidis*)* (BARROW, 1989; RODRIGUE et al., 1990). It is the most frequently isolated serovar in Europe as well as in America. Many cases of this disease have been associated with eating raw or undercooked eggs that were contaminated with Salmonellae. Broilers are also a considerable reservoir of infection for man (LISTER, 1988; ROBERTS and SOCKETT, 1994; LAX et al., 1995).

In the control of Salmonella infection in poultry there is a need to prevent intestinal colonization of the organism in chickens. Rapid establishment of intestinal microflora in young chicks increases their resistance to colonization by food-poisoning bacteria and provides a possible means of reducing Salmonella infection in commercial poultry flocks.

Under natural breeding conditions, the bird receives a complete flora from its parents or mates and from the environment. Under modern breeding conditions, clean hatching environment as well as separation of the chicks from their parents delays the establishment of the intestinal flora, thereby making the chick very susceptible to colonisation by transient pathogenic bacteria.

Competitive exclusion (CE) is one of the preventive measures introduced by NURMI and RANTALA (1973) whereby newly hatched chicks are pretreated by oral administration of suspensions or cultures of gut-content from suitable adult "donor" birds or by defined mixtures of intestinal bacteria. The validity of the CE concept is nowadays widely accepted, especially after WHO-FEDESA-FEP Workshop in 1994 (ANONYMOUS, 1994). There have been many reports on the experimental use of CE in poultry in many countries, and this concept is well established for non-invasive serotypes (GOREN et al., 1984; HIRN et al., 1992; WIERUP et al., 1992; STAVRIC and D'AOUST, 1993). A few studies have been carried out with invasive strains of *S.* Enteritidis (HINTON et al., 1991; NUOTIO et al., 1992; HUMBERT et al., 1997), but none with phage type (PT) 9a that is the most common pathogen type in Croatia (PRUKNER-RADOVČIĆ, 1998). In the present study a commercial CE product was given to newly-hatched chicks

raised under laboratory conditions to assess its efficacy in preventing colonization by pathogenic *S.* Enteritidis PT 9a strain.

Materials and methods

The initial birds. Newly hatched chicks were obtained direct from a hatchery. Day of arrival at the laboratory was designated “day 0”.

Two hundred and fifty one-day-old commercial AVIAN broiler chicks of mixed sex were used in the trial. Chick box paper liners as well as organs and meconium of 10 sacrificed chickens were cultured to screen for presence of Salmonellae. The remaining 240 chicks where allocated in two groups of 120 chickens and sixteen per group (total of 32) were separated and were used later as seeder birds.

Two replicate trials were carried out. Each group of chicks was housed in two fully protected rooms (2 tiers with 4 cages of 52 birds per tier), to which water and feed were applied *ad libitum*. Prior to use the feed (3 x 10 g of feed samples) was examined for Salmonellae using the standard method. Room lights were switched on continuously. Paper pads were placed on the bottom wire of the cages during the three weeks of life to promote spread of the challenge organism from seeder birds to the contact pen mates.

Application of CE product. Immediately after the chicks had been allocated to their respective groups the following treatments were administered:

Group 1 (challenge controls - 1C) - The chicks were individually dosed with 0.5 ml of physiological saline by gavage into the crop at day 0, day 1 and day 5.

Group 2 (treatment - 2T) - The chicks were individually dosed with 0.5 ml of diluted commercial CE product (4 g were diluted in 160 ml of water) by gavage into the crop at day 0, day 1 and day 5.

Cross-contamination between experimental groups during treatment was avoided by careful manipulation and protection of chickens and technicians.

Administration of challenge organism. On day 2, eight seeder birds per tier (2 per cage) (individually identified with wing tag) were challenged

by oral gavage with 0.5 ml of an approximate 10^7 organism/ml dilution of *S.* Enteritidis PT 9a strain (No E 47/1). The used strain was isolated from clinically diseased chickens in Croatia, carried 60, 47 and 36 MDa plasmids and was resistant to amoxycillin, ampicillin and carbenicillin. The organism was cultured in brain heart infusion broth (Becton Dickinson) at 37 °C for 18 hours. The seeder birds did not receive any of the experimental treatments.

Collection of samples and identification of Salmonellae. The fifth day after challenge and at 14 and 21 days of age, 12 chicks from each group were randomly selected and killed by cervical dislocation. The remaining unchallenged chickens were monitored for the five days. In that period of time they showed no clinical disease and were excluded from the trial. After opening the abdominal cavity, both caeca and spleen were removed aseptically. Caeca were placed in separate tubes containing 9 ml of buffered peptone solution (BPS). After careful mixing to obtain a reasonably homogeneous mixture, 0.5 ml of undiluted caecal suspension and 10-fold dilutions in BPS were prepared (from 10^{-1} to 10^{-7}) for surface plating (0.1 ml of each dilution) on brilliant green agar (Bio Merieux). Salmonellae were isolated from the spleens by direct streaking on brilliant green agar (Bio Merieux). The plates were incubated at 37 °C for 24 hours, when typical cherry-red colonies were counted as *Salmonella*. Randomly selected colonies were sub-cultured onto triple sugar iron agar (Becton Dickinson). Their identity was confirmed serologically by the plate agglutination method using either polyvalent or monovalent salmonella O and H antisera (Institute of Immunology, Zagreb, Croatia).

Seeders were removed at day 7 and Salmonellae were counted in the caeca according to the same procedure.

Statistical analysis. The four treatment groups were included in the analysis. The two replicates were combined for all statistical comparisons. The proportion of animals with caecal and spleen colonization was analyzed separately at each time-point, using Fisher exact test (FLEISS, 1981).

Treatment comparisons of the caecal *S.* Enteritidis titre at each time-point ($\log_{10}$ values) were first performed using parametric methods (analysis of variance). The factor included in the analysis was the treatment group. Additionally, an equivalent non-parametric method (Kruskal-Wallis rank

sum test) was also applied since some departures from the assumptions underlying the model of the analysis of variance could be expected. Therefore, non-normality of the error terms and non-constancy of the error variance was assessed by Wilk-Shapiro and Levene's tests, respectively (NETER et al., 1990).

Software and procedures. All statistical calculations were carried out using the SAS software (Release 6.12) on a Windows95/Pentium PC. The ANOVA, Kruskal-Wallis test and Fisher exact tests were carried out with the SAS "GLM", "Npar1way" and "Freq" procedures, respectively.

Results and discussion

Health of chickens. By means of bacteriological control, *Salmonella* Virchow was isolated from the paper in the transport boxes, but not from any of the cultivated organs. At the same time, bacteriological control of organs (liver, egg yolk) and meconium of 10 chickens were negative. Regardless of this unexpected finding, the trial was performed according to the previous schedule. Handling of chickens during the trial (gavage at age of 0 and 1 day, weighing) had no harmful effects with regard to the health of chickens. The 32 one-day-old chickens used as seeder birds had been successfully inoculated with *S.* Enteritidis PT 9a broth culture. Groups of eight of these chickens were placed in each of the tiers (2 per cage) on the second day and removed on seventh day. They were killed by cervical dislocation and the content of caeca was cultured, revealing *S.* Enteritidis in the maximal titer of 10^7 bacteria per 0.1 ml. In only three of 32 infected seeders (from two groups one, and once two) chickens *S*. Enteritidis was not confirmed. On average they were infected with *S*. Enteritidis in titer of $10^{4.03}$ organisms. There is no doubt that the seeders could infect chickens in the cages during the five-day period.

The effect of CE. Biostatistical analyses of isolation rate of *S.* Enteritidis from chicken caeca in trials are summarized in Table 1.

Statistical analysis revealed that on day 7 the proportion of chicken with *S.* Enteritidis in caeca was lower in the control group than in the trial group. All animals of the treated groups were infected, whereas 5 control animals remained uninfected. On days 14 and 21 the proportion of infected

Table 1. Proportion of chickens from the different groups with *S*. Enteritidis in caeca

Group	day 7	day 14	day 21
Challenge controls (1C)			
1C1	10/12	10/12	10/12
1C2	9/12	11/12	7/12
All	19/24	21/24	17/24
CE product (2T)			
2T1	12/12	5/12	4/12
2T2	12/12	7/12	2/12
All	24/24	12/24	6/24
P-values			
Fisher exact test	0.050	0.011	0.003

Table 1a. Average titer of *S*. Enteritidis in caeca of chickens from the different groups

Group	day 7	day 14	day 21
Challenge controls (1C)			
1C1	3.50 ± 1.93	3.25 ± 2.09	2.50 ± 1.57
1C2	2.42 ± 1.93	3.67 ± 1.83	1.58 ± 1.68
All	2.96 ± 1.97	3.46 ± 1.93	2.04 ± 1.65
CE product (2T)			
2T1	3.92 ± 0.90	1.83 ± 2.66	0.92 ± 1.44
2T2	4.17 ± 1.19	2.08 ± 2.35	0.58 ± 1.38
All	4.04 ± 1.04	1.96 ± 2.46	0.75 ± 1.39
P-values			
ANOVA	0.021	0.023	0.005
Kruskal-Wallis	0.063	0.019	0.004

animals was much lower in the CE treated group than in the challenge controls (both P-values <0.05). One-half of the treated animals remained infected on day 14 (12/24), and only one-quarter were still infected a week later (6/24). In the control group the proportion of infected animals reached 21/24 and 17/24 on days 14 and 21, respectively. Individual titers of *S.* Enteritidis in caeca of chickens in trials are summarized in Table 1a. Wilk-Shapiro tests of normality of residuals (P<0.05 for days 7, 14 and 21) and Levene's tests of equality of residual variance (P<0.05 for day 7) indicate that the analysis of variance could not be appropriate. However, both parametric and nonparametric methods led to remarkably similar results. On day 7 there was a trend to a significant difference (nonparametric method, P=0.063) or a significant difference (parametric method, P = 0.021) between treatments with respect to caecal colonisation. This difference reached a significant level (P<0.05 with both methods) on day 14 and a highly significant level (P<0.01 with both methods) on day 21.

Isolation rates of *S.* Enteritidis from spleen of chickens in trials are summarized in Table 2. From spleens of seven-day-old chickens, *S.* Enteritidis was not isolated, while at the age of 14 days it was found in group 2T1 in 5 and in 2T2 in 4 chickens. At the age of 21 days, spleens were positive from 2 chickens in group 2T1 and in 1 chicken from group 2T2.

There was no significant difference between treatment groups regarding the spleen colonization observed on days 14 and 21 (both P-values>0.1). However, as the proportion of infected animals in the control group was not modified between days 14 and 21 it was divided by 3 in the treated group. On day 14, the number of chickens with infected spleen was 4/24 in challenged controls and 9/24 in the treated group. On day 21 this proportion was 4/24 and 3/24, respectively.

The expected effect of CE is the colonization of chicken's digestive system that has not previously been exposed to microorganisms from the environment (NURMI and RANTALA, 1973). The same effect is expected in adult birds in which intestinal microflora was destroyed by continuous treatment with some antibiotics (WIERUP et al., 1992; STAVRIC and D'AOUST, 1993). According to the results of previous investigations, and practical use of microorganisms contained in CE products, colonization of guts will

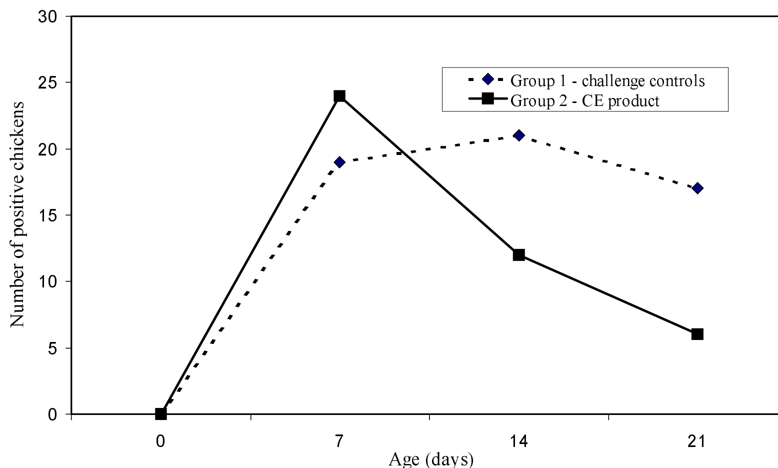

Fig. 1. Finding of *S.* Enteritidis in caeca of chickens

prevent the negative effect and continuous colonization of pathogenic microorganisms such as salmonellae, colibacteria and others (SEUNA et al., 1980; CORRIER et al., 1993; STAVRIC and D'AOUST, 1993). Nevertheless, it remains an open question as to whether highly pathogenic bacteria can overwhelm the resistance of a digestive system provided by colonization of favorable microflora. In previous investigations, where commercial CE product was applied to newly hatched chickens by means of spraying or *via* drinking water, bacteria for challenge were *S.* Enteritidis or *E. coli* of moderate pathogenicity (GOREN et. al., 1984). Compared to this we chose a challenge with a pathogenic strain of *S.* Enteritidis PT 9a (PRUKNER-RADOVČIĆ, 1998) using shedders (8 per each experimental group) that had been successfully infected. This infection was confirmed by determined average bacterial titers of $10^{4.03}$ *S.* Enteritidis in 0.1 ml of caecal contents. In period from day 2 to day 7 (five days in total) all chickens had ample opportunity for exposure to seeders of *S.* Enteritidis. At the age of 7 days the infection rate was between 79% (Group 1C) and 100% (Group 2T) of chickens. Although at the 7th day the control group had significantly more colonies than the treated groups, its biological significance is doubtful.

At the age of 14 days, in control chickens that did not receive CE product the level of contamination increased by 8.34% and rose to 87.50%, which was not significant. In relation to this, in group 2T (received CE product at 0, 1 and 5 days) 50% of the chickens were infected, which is significantly less than at the age of 7 days. At the same time the titer of *S.* Enteritidis in intestinal content was only slightly lowered (from $10^{4.04}$ to $10^{3.92}$).

As shown in Table 2, *S.* Enteritidis always infected fewer spleens than intestines. The biggest difference was in the control group of chickens at the age of 14 days, when 21 intestines and only 4 spleens were infected. At the age of 21 days there were still 17 positive intestines and the same number of positive spleens (4). From only one chicken of 1C1 group, at the age of 21 days, *S.* Enteritidis was not isolated simultaneously from spleen and intestine.

At the age of 14 days in 2T groups, *S.* Enteritidis infected 9 spleens and 12 intestines. A week later, on the 21st day, there were still 6 intestines positive, 3 of which were paired with infected spleens. At 14 days only 4 of 9 chickens had simultaneous infection. At the 21st day of age, in two

Table 2. Proportion of chicken from the different groups with *S.* Enteritidis in spleen

Group	day 14	day 21
Challenge controls (1C)		
1C1	3/12	3/12
1C2	1/12	1/12
All	4/24	4/24
CE product (2T)		
2T1	5/12	2/12
2T2	4/12	1/12
All	9/24	3/24
P-values		
Fisher exact test	0.193	>0.999

chickens *S.* Enteritidis infected both intestine and spleen, while in 1 chicken of 2T2 group the finding was positive only in spleen. It is obvious that, starting from the 14th day, an intestinal infection had begun to spread systemically and with a higher incidence in the chickens receiving the CE product. Intestinal infections were reduced in all chickens between days 14 and 21.

Conclusions

It can be concluded that the CE product provided a significant protective effect against pathogenic *S.* Enteritidis used for challenge. Undoubtedly, by its activity - which was confirmed by comparison with control chickens - the level of infection was significantly reduced regarding the number of infected chickens as well as the number (titer) of *S.* Enteritidis isolated from sacrificed chickens. Based on observations made on days 14 and 21 caecal colonization with *S.* Enteritidis was significantly reduced by CE treatment. At the same time, with regard to spleen colonization there was no significant difference between the treated and untreated groups. The rate of spleen infection was fairly low in all groups. The relatively reduced efficacy of CE was therefore attributed to the pathogenicity and the high dosage of *S.* Enteritidis used for challenge in the experiment. The high level of contamination used is unlikely to occur in natural field conditions.

It seems reasonable to perform further experiments of prolonged duration (up to 42 days) which is the average length of fattening for commercial broilers. The aim of any mitigation strategy for salmonella control, here *S.* Enteritidis, and layers/broilers, should be salmonella-free layer/egg and broiler chickens. This means that in investigating the effect of a CE product it should be investigated under conditions in which a slower infection process is duplicated, seeder birds seeded at lower level, and strains differing in virulence being used for challenges.

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SAŽETAK

Ukupno 250 netom izleženih tovnih pilića korišteno je u dva usporedna istovjetna pokusa. Pilićima je u preporučenoj dozi primijenjen komercijalni proizvod za kompetitivnu ekskluziju (CE) u dobi 0, 1 i 5 dana (pokusna skupina) ili fiziološka otopina (kontrolna skupina). Drugog dana, 16 pilića bilo je zaraženo bakterijom *Salmonella* Enteritidis fagotipom 9a (suspenzijom s približno 10^7 bakterija/ml). Ti pilići bili su upotrijebljeni kao izvor zaraze i smješteni zajedno s ostalim pilićima pokusne odnosno kontrolne skupine. Petog dana nakon zaražavanja, tj. u dobi 14 i 21 dan, izdvojeno je 12 pilića po skupini kojima je određen broj salmonela u sadržaju cekuma. Udio zaraženih pilića bio je značajno manji ($P<0,05$) 14. i 21. dana u skupini koja je primila proizvod CE u odnosu na kontrolnu skupinu. Od ukupnog broja pilića pokusne skupine, u 50% dokazana je infekcija 14. dana, dok se 21. dana taj broj smanjio na četvrtinu. Udio zaraženih pilića u kontrolnoj skupini iznosio je 21 odnosno 17 u odnosu na 14. i 21. dan. Salmonela je bila izdvojena 14. dana iz slezene 4 pileta kontrolne te iz 9 pilića pokusne skupine. Taj udio 21. dana iznosio je 4/24 u odnosu na 3/24. Ovim istraživanjem dokazan je znatan zaštitni učinak kompetitivne ekskluzije u sprečavanju zaraze patogenim sojem bakterije *Salmonella* Enteritidis fagotip 9a.

Ključne riječi: *Salmonella* Enteritidis, kompetitivna ekskluzija, pilići, salmoneloze

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