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SALMONELLA ENTERITIDIS ISOLATION FROM BROILER CHICKENS INFECTED WITH LOW DOSES

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In this paper we present the possibilities for detection of Salmonella enteritidis (SE) by bacteriological and serological technics. Also, we studied which tissue is the most suitable sample for Salmonella isolation. One day old and three weeks old broiler chickens were experimentally infected with low doses of SE. For the isolation of SE we used Rappaport Vassiliadis media. Specific antibodies in sera were detected using ELISA. According to our results, broiler chickens were susceptible to infection with low doses of SE. However, specific antibodies could not been found. SE was isolated from caeca, but not from livers of infected chickens when tested at the end of the experiment (at 6 weeks). Our results revealed that bacterial cultures are still the method of choice for detection of SE in broilers flocks.

Key words: Salmonella enteritidis, broiler chickens, experimental infection, bacteriology, serology.

INTRODUCTION

Since 1990 *Salmonella enteritidis* has become pandemic and is the most important cause of a food-borne disease associated with poultry products. The outbreak of salmonellosis in humans arises most often after consumption of chicken eggs or poultry products (Rabsch *et al*., 2001). There are also reports of the disease caused by consumption of barbecued chicken meat (van de Giessen *et al*., 1992). Contamination of chicken meat during processing in abattoirs and the discovery of *Salmonella* in chickens from retail stores is significant (Uyttendaele *et al*., 1998, Capita *et al*., 2003). Consequently the interest in the eradication of Salmonella from poultry farms and processing facilities has arisen and is one of the major problems in poultry industry. Hazard analysis of critical control points (HACCP) has been developed thus enabling veterinarians to work more creatively in the search of salmonella organisms in egg and meat products. Monitoring was introduced in farms (Gast *et al*., 2002, Cooper *et al*., 1989, Furrer *et al*., 1993) and new-sophisticated bacteriologic media were discovered to enhance salmonella isolation (Vassiliadis 1983, Hammack *et al*., 2001).

It has been proposed that the bacterial count can influence the development and duration of the immune response to *Salmonella* as well as *Salmonella* excretion (Humphrey *et al*., 1991). Also, it was shown that infection of one day old

chickens can lead to prolonged *Salmonella* shedding (Gast and Beard 1989) and that early infected chickens can carry *Salmonella* until maturity (Gast and Holt 1998). Since serology tests are proposed together with cultivation of bacteria, for the detection of *Salmonella* in poultry flocks challenged broiler chickens with low number of SE in broiler chickens in order to estimate the value of both methods. The reason for this was threefold. First, chickens infected with high doses of *Salmonella spp* usually suffer from significant morbidity and mortality and bacteria is easily isolated in such circumstances while antibodies are found in high titers. Second, in our country we rarely have a chance to recognize and diagnose such clinical Salmonellosis and finally *Salmonella* differ in their immunogenicity so not all strains will induce a measurable humoral immune response. Therefore bacteriology examination was performed by Rappaport Vassiliadis media, a recommended medium for detection of a low number of the *Salmonella* (Hammack *et al*., 2001) and serology testing was done by ELISA.

Also, our goal was to determine, which organs (cecum versus liver) would be the best choice tissue to isolate *Salmonella* in chickens infected with low doses. Our results revealed that after inoculating chickens with a low number of SE, the laboratory cultivation procedure has an advantage over serology tests, even in circumstances where sensitive tests, such as ELISA, are performed.

MATERIAL AND METHODS

Experimental chickens

Sixty commercial one-day old broiler chickens were purchased from the local hatchery on two occasions. First, thirty chickens were raised in the experimental unit of the Institute until the age of 3 weeks, when they were selected into a group of 20 chickens to be subsequently infected. The remaining 10 uninfected chickens served as controls and were held separately. When the first batch of chickens was 3 weeks old a new batch of 30 one-day old chicks was delivered from the same local hatchery. On arrival 20 chicks were selected for Salmonella infection while 10 unifected control chicks were kept in a separate room.

Preparation of Salmonella enteritidis for inoculation

Salmonella enteritidis was previously isolated from broiler chickens, and grown overnight in peptone water at 37°C. Next day SE was diluted in sterile phosphate buffered saline pH 7.4 to reach 2×10^3 and 2×10^5 cfu/ml for inoculation of one-day old and 3 week old broilers respectively.

ELISA test

ELISA was performed with a commercial kit purchased from Guldhay (Biomedica Gruppe). The plates were coated with the SE lipopolysaccharide (LPS) antigen. Positive and negative control sera were ready to use without dilution, while test sera had to be diluted at a ratio of 1:500. When negative and control sera, as well as diluted sera from the experimentally infected chickens,

were placed in 96 wells, the plate was covered with an adhesive cover and was incubated at 37°C for 30 minutes. The plates were washed with diluted washing solution and enzyme conjugate reagent was added to each well. After additional 30 minutes of incubation at 37°C the plates were washed again and the substrate reagent was added to the plates. The plate was covered with an adhesive cover placed at 37°C for 15 minutes after which a stop solution was added. The plates were read at 550nm on a Microtitre plate reader.

Bacteria isolation procedure

Cloacal swabs were cultivated in selenite broth overnight at 37°C. The next day 0.1ml of selenite broth was transferred to Rappaport Vassiliadis medium and cultivated at 42°C for 24 hours. A full loop was transferred to Brilliant Green media and after 24 hours of incubation at 37°C suspicious colonies were transferred to triple sugar agar (Kligler) at 37°C for 24 hours. *Salmonella enteritidis* was confirmed on the base of biochemical characteristics and finally diagnosed after slide agglutination with sera against somatic and flagellar antigen.

Experimental design

Ten out of the 20 one-day old chickens (D-1) were infected per os with 10^2 cfu/0.1 ml of *Salmonella enteritidis* strain (marked as A) and additional ten chickens were placed together with the infected group as contact chickens (marked as C). Ten chickens were held separately as controls. Another group of ten 3 week old chickens (W-3) was inoculated with 10^4cfu/0.1ml of SE (marked B) and additional ten chickens of the same age were placed with this group as contact chickens (marked D). In separate rooms ten one-day old and ten 3 week old chickens were held as uninoculated controls.

Therefore this experiment consisted of the following experimental groups:

Ten one-day old chickens infected with 10^2 cfu/0.1 ml (marked A) and ten contact exposed chickens (marked C).

Three week old chickens infected with 10^4 cfu/0.1ml, (marked B) and contact exposed chickens of the same age (marked D).

The controls were one day old and 3 weeks old chickens that were not infected and were kept separately in two separate rooms in the experimental facilities at the Institute.

At day 5,7,14 and 21 average body weights were estimated in groups A, C, B and D as well as in the control groups. Fourteen days in a row cloacal swabs were taken from experimentally infected chickens and their contact counterparts. From the controls cloacal swabs were taken on day 1 and at the end of the experiment.

Antibody titers were measured in groups A and C at 3, 4, 5 and 6 weeks after infection and in group B and D at 2 and 3 weeks after infection. In the controls antibodies were determined when the chickens were 6 weeks of age.

At the end of the experiment (at 6 weeks), after sacrificing the birds swabs from the ceca, liver and cloacal were taken from chickens infected by contact (group C and D).

Statistical analysis

All data were statistically processed using Anova. Regression analysis was performed by Dunett and LSD test.

RESULTS

During the experiment, two chickens died in group A and four chickens in group C. Salmonella was confirmed in two out of the six chickens.

Chickens infected at day one and those infected at 3 weeks of age with a low number of SE had lower body weight compared to the controls (Table 1 and 2). Lower body weight in chickens infected with SE was present until the end of the experiment (data not shown).

Table 1. Body weight of chickens infected at one-day of age with 10^2 cfu/0.1ml, chickens infected by contact and control uninfected chickens

Days after infection	BW of chickens infected at one-day of age		Body weight of contact chickens		Control non infected chickens	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
D-5	76.31	20.81	77.88	20.46	86.22	7.29
D-7	121.67	39.23	135.29	12.65	143	17.15
D-14	330[b]	48.99	300	85.58	367[a]	34.28
D-21	671[b]	72.0	690[c]	77.71	755[a]	41.05

D-14, b lower than a, $p < 0.05$
D-21, b lower than a, $p < 0.05$
c lower than a, $p < 0.05$

Table 2. Body weight of chickens infected at 3 weeks of age with 10^4 cfu/0.1ml, chickens infected by contact and control uninfected chickens

Days after infection	BW of chickens infected at 3 weeks		Body weight of contact chickens		Control non infected chickens	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
D-5	687	56.98	635[b]	71.52	696[a]	48.12
D-7	871	71.01	825	88.06	902	74.09
D-14	1360[b]	104.98	1314	111.97	1455[a]	93.24
D-21	1971	163.12	1985	162.87	1928	145.75

D-5, b lower than a, $p < 0.05$
D-14, b lower than a, $p < 0.05$

Salmonella enteritidis was isolated from cloacal swabs in 30% of chickens infected with 10^2cfu/0.1ml (marked A) and in 90% of chickens that were infected by contact (marked C). In the group of chickens which were infected orally with 10^4 cfu/0.1ml (marked B) SE was isolated in 66% of the birds and in 100% of contact exposed chickens (marked D), Fig 1.

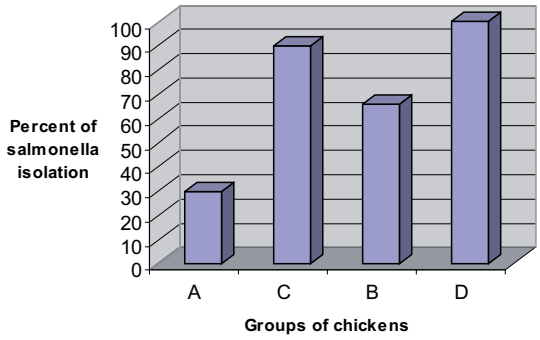

Figure 1. Incidence of *Salmonella* isolation from cloacal swabs during the whole experiment

Since contact exposed chickens (marked as C and D) had the highest incidence of Salmonella, confirmed by cultivation of cloacal swabs taken during the experiment at the time when the birds were sacrificed (age 6 weeks), the cloacal swabs were taken once more and ceca and liver samples were submitted to isolation of bacteria using highly selective media. This was done in order to find out which samples (ceca, swabs or liver) are preferable for *Salmonella* isolation. The results summarized in Fig 2 clearly show that the best tissue was ceca, since *Salmonella* was frequently found in this organ when chickens infected with a low number of this organism, reached 6 weeks of age. In the livers of those birds *Salmonella* could not be found at the end of the experiment.

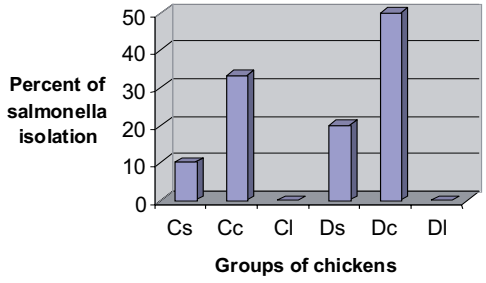

Figure 2. Rate of *Salmonella* isolation from cloacal swabs (Cs and Ds), ceca (Cc and Dc) and liver (Cl and Dl) 3 weeks post infection

In the group of chicks infected at one-day of age and in their contact counterparts (group A and group C) at 3, 4, 5 and 6 weeks following infection, after performing the ELISA test, specific antibodies to SE were not found. The same result was in 3 week old infected chickens and their contact counterparts, when antibodies were measured at 6 weeks of age when the experiment was concluded.

DISCUSSION

Data from this experiment show that commercial broilers, purchased from the local hatchery, were susceptible to infection at one day of age (D-1) as well as at 3 weeks (W-3) when they were infected with very low doses of SE. Interestingly, in both groups of chickens (D-1 and W-3) the contact exposed birds have shown higher rate of *Salmonella* isolation implicating that this organism spreads very quickly among the chickens. Also body weight of infected chickens was lower compared to the control groups, this was also an indicator of recent infection. Air, feed, droppings or infected litter can transmit salmonella from bird to bird and this occurs within hours. Bailey *et al*. (1994) found that broilers, which are in close proximity to the hatchery cabinet, can be easily cross-contaminated even if one single contaminated egg is present. According to Cox *et al*., (1990) as few as 100 cfu of *Salmonella thyphimurium* given through various body orefices is sufficient to infect broilers which will continue to actively excrete *Salmonella* during grouth. Experimental data confirm such findings since one-day old chicks, which received only 10^2 cfu, transmitted the infection to their contact counterparts. Also chickens infected at W-3 with 10^4 cfu of SE continued shedding *Salmonella* to the contact exposed group. Although, given in a small number SE could be detected in most of the chickens until the end of the experiment, suggesting that SE did not clear from the intestinal tract. Barrow *et al*., (1988) found differences in the duration of Salmonella colonization among the three strains that were tested. *Salmonella thyphimurium* and *S. menson* could be detected in the cecal content 34 days while *S. cholere suis* could be detected in the alimentary tract up to the 14^{th} day post infection. From this experiments it is clear that SE used as a challenge would most likely persist and circulate in a poultry flock until slaughter. In such circumstances and in spite of the fact that some of the chickens died (group A and C) and most of the chickens excreted SE through feces (confirmed by *Salmonella* isolation from cloacal swabs), anitibodies were not found during 6 weeks in groups A and C or after 3 weeks in groups B and D. The only explanation for such findings is that a low number of *Salmonella* cells creates a situation where antigen concentration is not sufficient to initiate antibody production. However, it is possible that chickens could became antibody positive after 6 weeks at the time we ended the experiment. Therefore, from the present work, the question arises whether serology control by ELISA test can be beneficial in broiler production since not all chickens are able to develop desirable antibodies before slaughter. This test is very effective in controlling SE infection of laying hens and should not be omitted. In the previous research we detected antibodies in few flocks of layer hens even in circumstances were SE was not confirmed during bacteriology examination

(Velhner *et al*., 2004). Considering the possibility for the presence of other SE serotypes in broilers and data from these experiments it is the best to relay on bacteriology testing in controlling *Salmonella* in broiler flocks.

Our second goal was to test which organs are best choice samples for *Salmonella* isolation. For many years we have tested liver in a routine examination from all types of chickens at any age period. Other researchers in their experimental work have examined ceca for the presence of *Salmonella* (Marion Duchet-Suchaux *et al*., 1997, Kaiser and Lamont 2001, Gast and Holt 1999). Indeed, our results clearly show that ceca from chickens infected with low doses of *Salmonella* is the most suitable organ for *Salmonella* isolation. At the end of this experiment SE could not be found in livers, while in chickens infected by contact exposure SE could be isolated from ceca in 30% and 50% of chickens in groups C and D respectively. *Salmonella* hides in the intestinal tract of chickens, multiplies and excretes through feces in the environment. We strongly recommend that in every routine bacteriology investigation ceca must be controlled for the presence of *Salmonella* and that such control should be performed also on highly sensitive media such as Rappaport Vassiliadis used in this work.

Overall results from these experiments show that broilers infected by contact experienced a higher rate of *Salmonella* infection (according to mortality, body weight and SE isolation). This leaves a space to speculate that after challenging one-day old and 3 week old chickens SE multiply in the intestine to a high level and transfer SE infection to chickens by contact. Gast and Holt (2001) similarly found that the passage of SE through lying hens enhances the incidence of *Salmonella* in layed eggs. Weather this is truly happening in nature is difficult to state, but it is certain that *Salmonella* easily spreads in the environment and after reactivation caused by stress, for instance, can infect many birds. *Salmonella enteritidis* that was used as a challenge strain in these experiments was invasive but was not immunogenic when given in low numbers. Apparently, *Salmonella* serotypes differ in their immunogenic potential which leads us to the conclusion that serology tests *per se* would not be the best choice to control SE organisms in broilers.

In conclusion we found that one-day old and 3 week old commercial broilers are highly susceptible to SE infection even at very low doses. Chicken caeca are the most suitable organ for *Salmonella* isolation when tested at the end of the experiment. In ELISA test specific antibodies to SE were not detected probably because the inoculum was too low, therefore to explore the possibilities of application of ELISA in broiler industry more research is needed. Finally, highly selective media are desirable in order to discover small numbers of *Salmonella* in chicken organs and feces.

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IZOLACIJA *SALMONELLA ENTERITIDIS* IZ BROJLERSKIH PILIĆA NAKON INFEKCIJE MALIM DOZAMA

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SADRŽAJ

U ovom radu su prikazani rezultati ispitivanja mogućnosti detekcije *S. enteritidis* (SE) primenom bakterioloških i seroloških tehnika i pogodnosti pojedinih organa za izolaciju salmonela. Jednodnevni i tronedeljni brojlerski pilići su bili veštački inficirani malim dozama SE a za izolaciju SE iz jetre, cekuma i kloakalnih briseva korišćena je podloga Rappaport Vassiliadis. Zatim je primenom ELISA tehnike utvrđivano prisustvo specifičnih antitela u krvi pilića. Brojlerski pilići su bili podložni na infekciju malim dozama SE, međutim, specifična antitela nisu utvrđena. SE je izolovana iz cekuma pilića, ali ne i iz jetre (na kraju ogleda). Naši rezultati ukazuju da je bakteriološka kultivacija još uvek metod izbora za detekciju salmonela u brojlerskim jatima.