

**INFLUENCE OF SALMONELLA INFECTION IN CHICKENS ON THE OUTCOME OF CAMPYLOBACTERIOSIS IN EXPERIMENTAL CONDITIONS**

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*Meat, table eggs and their products are very important in human nutrition. Therefore, it is necessary to monitor the health status of commercial flocks as much as the quality of poultry products in the food chain. Campylobacter sp. and Salmonella sp. are widely distributed in nature. The influence of these bacteria on animal health depends on the immune response. If animals are not immunologically compromised, the infection is latent and clinical symptoms are absent. Unlike animals, these bacteria cause serious diseases in humans and the morbidity is quite high. The main transfer of infection to humans is via poultry products. The goal of this work was to study the role of Salmonella in artificially infected chickens onto the outcome of clinical campylobacteriosis. It is certain that salmonella infection in poultry damages the immune system of chickens, enabling Campylobacter to multiply and subsequently induce a disease. Three groups of chickens were included in the experiment. The first group received a suspension of field strain of Campylobacter jejuni (C. jejuni) and Salmonella enterica serovar Enteritidis (S. Enteritidis). The second group received an inoculum prepared from the field isolate of Campylobacter jejuni and the third group received the field isolate of Salmonella enterica serotype Enteritidis, only.*

*In artificially infected chickens Campylobacter and Salmonella were confirmed by isolation and identification according to morphological, cultural and biochemical properties. Humoral immune response of infected chickens was monitored using the complement fixation test (CFT). In chickens infected with C. jejuni and S. Enteritidis the clinical symptoms were recorded. The results from this experiment show that salmonella infection damages the immune system of the chickens enabling Campylobacter to alter the health status of the host.*

*Key words: Campylobacter jejuni, campylobacteriosis, immune response, Salmonella enteric serovar Enteritidis*

## INTRODUCTION

The intensive agricultural industry, faster and improved transfer of goods, including transport of humans and animals contribute to the widespread nature of *Salmonella* and *Campylobacter*. The life style changes and animals are raised in close proximity to humans (for pleasure, safety and recreation reasons) so these bacteria, find an easy way to humans. According to Saif (2003) epidemiological data have shown that bacteria, such as *Salmonella* and *Campylobacter*, do not cause characteristic clinical symptoms in animals, or the symptoms are totally absent. The experience of Otasević *et al.* (2000) implies that *Campylobacter* may cause a disease, but also, in many cases, even when the bacteria were confirmed in the laboratory, clinical symptoms were absent. In most of the cases a relationship between *Campylobacter* isolated from susceptible species and the occurrence of disease cannot be proven. In *Salmonella* the percent of morbidity and mortality does not exceed 10% in animals (Saif, 2003). On the contrary, salmonellosis is a zoonosis, characterized by high morbidity, sometimes even up to 100% to humans. From the epidemiological point of view salmonella infection creates a problem since animals and animal products present a source of infection in humans. Salmonella can produce toxins and indirectly disturb normal homeostasis predisposing the organisms to other unfavorable factors and infections caused by other microorganisms. Antonijević *et al.* (2001) and Saif *et al.* (2003) stated that during salmonella infection the immune response is weakened. In their experiments it was proven that lymphocytes were depleted from lymphatic organs and atrophy was described, as well. Such limited immunosuppressive effects of *Salmonella* contributes to persistent infection. Similar results were found in the research of Hassan and Curtis (1994). The authors examined the impact of *Salmonella* on lymphatic organs in chickens, and shedding via feces and have shown that lymphocytes were depleted, the lymphoid organs atrophied and subsequent immune suppression was induced. These changes were transient and depended on the size of inoculum. *Salmonella* shedding in feces lasts longer in chickens infected with field strains, probably as a result of a weakened immune system.

According to the aforementioned data on *Campylobacter* and *Salmonella*, and having in mind its significance the objective of our research was to detect if salmonella infection in chickens can have detrimental effects on the immune system and how it affects its functioning.

## MATERIAL AND METHODS

One day old chickens of Arbor Acres provenience were obtained from the local hatchery. They were raised at the experimental units of the Institute. All the chickens were tested on the presence of *Salmonella* and *Campylobacter* before the beginning of the experiment. The experiment was carried out when the birds reached 14 days of age.

### Material

*C. jejuni* and *S. Enteritidis* were used to infect chickens in the experiment. The chickens were divided into three groups. Each group consisted of 30 birds. The first group was infected *per os* with a suspension of filed isolates of *C. jejuni* and *S. Enteritidis*. The second group received 0.5 mL of  $1 \times 10^8$  cfu/mL of the *C. jejuni*, and the third group received 0.2 mL of  $1 \times 10^8$  cfu/mL of *S. Enteritidis* (field strain).

The chickens were sacrificed at 10, 17 and 22 days post infection. For bacteriological examination the liver, spleen and caecum, as well as the distal part of the intestine were used.

The selective media: Peptone water, modified Rappaport Vassiliadis agar, Salmonella differential agar, McConkey agar and Triple sugar agar were used. For biochemical

identification for *Enterobacteriaceae* the API20E strips (BioMerieux, France) were available. *Campylobacter jejuni* was isolated on Columbia agar with 5% sheep blood and Campyloset antibiotic suspension (BioMerieux, France). The suspension contained the following

antibiotics: cefoperazone 3 mg, colistin 2000 U, vancomycin 2 mg and amphotericin B 0.4 mg. The amount of 2 mL of the antibiotics was added to 100 mL Columbia agar. After inoculation the medium was incubated in a McIntosh jar with gas pak (BioMerieux, France) to provide microaerophilic conditions.

API Campy strips were used for biochemical identification of *Campylobacter jejuni*, (BioMerieux, France). For serology the blood samples were taken 7 days post infection and every seven days thereafter.



Figure 1. The procedure of artificial infection of chickens

### Methods

#### *Clinical investigation*

The animals were held in the experimental units at the Institute and were observed on daily basis. Diarrhea was recorded when dirty feathers on the chest and cloaca appeared.

#### *Pathological observation*

In sacrificed chickens pathological changes in intestine and liver were noted. Changes on the liver and intestines were followed by pathoanatomical examination.

#### *Isolation of Salmonella Enteritidis*

The cloacal swabs were taken from the chickens from experimental Groups 1 and 3. The parenchymatous organs from the sacrificed chickens were examined



Figure 2. Red color on (RajHans Medium) means *Salmonella* findings

on the presence of *Campylobacter jejuni*. Cloacal swabs were placed in tubes with peptone water, while the liver, spleen and intestine were placed in Erlenmeyer dishes with 50 mL of peptone water. Peptone water was incubated at 37°C for 24 hours for pre-enrichment purposes. One milliliter of peptone water was transferred to semisolid media Rappaport Vassiliadis (HiMedia) according to the recommendation of Quinn *et al.* (2002).

Incubation of the semi solid media was done at 42°C for 24 hours. After overnight cultivation the full loop was transferred to Salmonella differential agar (RajHans Medium) (HiMedia) and McConkey agar (Torlak, Belgrade-

Serbia). All the bacteria red in color on RajHans media, or in the color of the plate in McConkey, were inoculated to triple sugar agar (Torlak, Belgrade-Serbia). Triple sugar agar was cultivated overnight at 37°C. Colonies suspected to be *Salmonella* were subjected to biochemical tests. Serological typing was additionally carried out with polyvalent (D) and phase (gm) sera, according to Quinn *et al.* (1998).

#### *Isolation and identification of Campylobacter jejuni*

The samples were inoculated on the adequate media and placed in a jar with gas pack system. The media was incubated at 37°C and 42°C for 48 hours. After incubation suspect colonies were prepared for microscopic examination i.e. Gram staining. Gram negative spiral oxidase positive microorganisms were identified by biochemical procedures, applying API Campy system and accompanied software program (BioMerieux), (Quinn *et al.*, 2002; Biberstein and Zee, 1990). The enzymatic activities of *Campylobacter jejuni* was done by examining urease, reduction of nitrates, esterase, hipurate, gamma-glutamyl transferase, reduction of chloride to triphenyl tetrazolium, pyrrolidonyl arylamidase, L-arginine arylamidase, L-aspartate arylamidase, alkaline phosphatase, production of H<sub>2</sub>S, assimilation of glucose, succinate, acetate, propionate, malate and citrate. Also, the sensitivity to nalidixic acid, cefozoline and erythromycin was examined.

#### *Serology investigation*

The specific antibody titer to *C. jejuni* was determined with the complement fixation test applying a commercial kit produced by Virion, Switzerland. The blood samples were taken just before the infection and every seven days thereafter.

Statistical analysis was done by variance analysis. The specific antibody titer in the blood sera of the experimental animals was expressed as percentage.

## RESULTS AND DISCUSSION

Before the experiment it was important to determine whether the chickens are salmonella and campylobacter free, and that they have no specific antibodies against *C. jejuni*. During the control examination specific antibodies against *C. jejuni* were not detect.

*C. jejuni* was found in the intestine of chickens infected with either *S. Enteritidis* and *C. jejuni* or infected only with *C. jejuni*. The infection was therefore successful and the intestines were colonized with *C. jejuni*. According to Saif *et al.* (2003) infection of the chickens at the age of 12 hours induces diarrhea, while infection of the chicks when 3 days old with  $10^9$  CFU does not induce diarrhea. If diarrhea occurs, it is usually 6 hours after infection and lasts for 10 days. The clinical symptoms tend to appear depending of the strain, number of bacteria, stress and immunosuppression. The distal parts of the intestines are usually affected (jejunum and cecum), after *C. jejuni* infection. Pathological changes are not characteristic and are described as enlargement of the intestine, as a consequence of accumulation of the water and gelatinous content inside the intestine. Hemorrhages are not always present during infections caused by *Campylobacter*. Kazwala *et al.* (1992) showed that colonization of the intestine depends on the age of birds and the size of inoculum (cfu/mL). Young species are more susceptible to infection with *C. jejuni* since colonization is easier. The same authors state that higher inoculum size of *C. jejuni* induces more prominent clinical symptoms. Diergaardt *et al.* (2004) reported that 100-500 units/mL of *Campylobacter* are necessary to obtain clinical symptoms in experimental conditions.

In this experiment the soiled feathers around the cloaca were noticed, as well as watery diarrhea in the first two weeks after infection. Blood traces were found in feces to a lesser extent. Foamy feces with traces of blood (Fig. 3) were noticed on the litter. In the experimental group of chickens infected with *S. Enteritidis* no clinical symptoms were found.

Our research indicated that clinical symptoms in experimental birds, when simultaneously infected with *C. jejuni* and *Salmonella* strains are similar to the infection caused by *Campylobacter*, in spite the fact that watery diarrhea was not found consistently in experimental chickens. Such a finding was absent in the group of chickens infected with *C. jejuni* only, and a group of chickens infected with *S. Enteritidis*.

According to data from the available literature such symptoms are found in chickens subjected to stress. The clinical symptoms can be attributed to biological stress caused by salmonella infection, since in such circumstances the immunological system is disturbed and immunodeficiency develops. In these conditions clinical and pathomorphological manifestation of campylobacteriosis is possible, which was confirmed in our experiment.

Mixed infection was also investigated by Zweifel *et al.* (2004). They found that a simultaneous infection with shiga toxin producing *E. coli*, *Salmonella* spp and *Campylobacter* spp in sheep, contribute to the development of clinical symptoms, that were not apparent in laboratory differentiation. In this experiment

red and yellow spots on the liver were not found in chickens that had clinical campylobacteriosis, but the watery and foamy diarrhea was found only in the group of birds infected with *Campylobacter* and *Salmonella*. *Salmonella* caused stress and immuno-suppression.

In chickens that did not have characteristic pathological findings in the liver after infection with *C. jejuni*, antibodies were found and *C. jejuni* was confirmed by bacteriological examination. Obviously, antibodies can be found in chickens that develop clinical symptoms and even if they do not become ill. This is similar in



Figure 3. Feces of infected chicken



Figure 4. Cecum filled with foamy hemorrhagic content

both animals and humans. According to Janvier *et al.* (2000) in healthy people antibodies to *C. jejuni* can be found in 30% of cases. Antibody titer higher than 1:8 was considered positive. Antibody titer to *C. jejuni* in blood sera of the chickens from the first and second group ranged from 1:4 to 1:8, three weeks after infection. In the fourth week of the experiment the antibody titer ranged from 1:4 to 1:16.

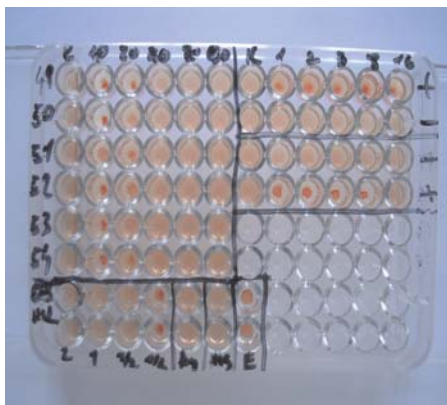
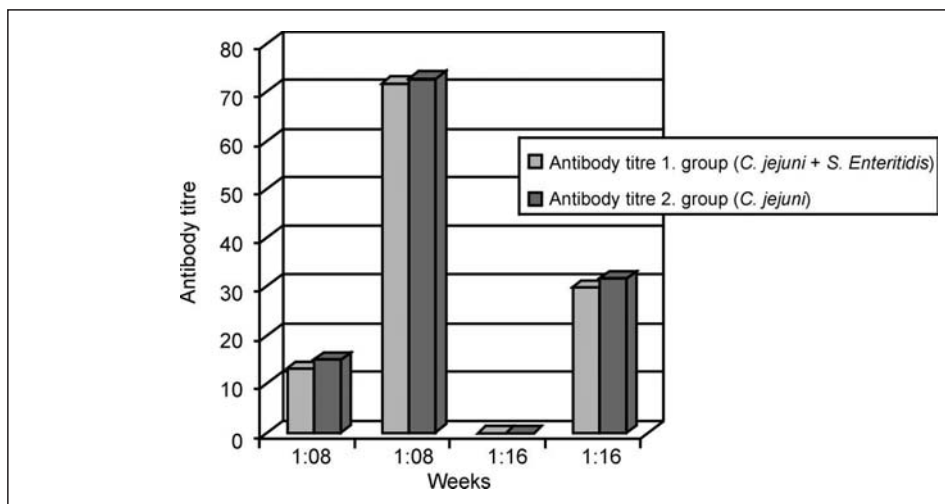


Figure 5. CFT in micro-well plate

Statistically significant differences were not found between experimental groups. However, significant differences were noted comparing the obtained specific antibody titers against *C. jejuni* (95%) between some experimental groups in different weeks of the trial. The differences were noted between groups 1 and 2. When antibody titers were compared to the first experimental group (infected with

*C. jejuni* and *S. Enteritidis*) and to the second group (infected with *C. jejuni*), it was found that the specific antibody titers were higher in the second group comparing to the first one, in the 5<sup>th</sup> week of the experiment. Mixed infection with *Salmonella* and *Campylobacter* affects the immune response hence was better in the group of chickens that received *C. jejuni* only. Mixed infection provoked disturbances in lymphocytes in the last week of the experiment and, subsequently, the antibody titer was lower.

According to Saif et al. (2003) salmonella infection can affect the host in different ways. Besides the well known affect on overall health status (from the aspect of colonization, invasion and pathogenesis), the impact on the immune system is important. A depletion of lymphocytes and atrophy of immune organs, as a sign of weakening of the immune system, can occur in salmonella infected chickens. The transient nature of immune-suppression in salmonella infected animals will contribute the development of a persistent infection and other pathogenic bacteria tend to persist in the intestines. In order to determine whether immunosuppression occurs after salmonella infection, a similar investigation was done by Hassan and Curtiss (1994). They tried to find out how the infection with the pathogenic or vaccine strains of *Salmonella* affects chicken immune respond and how, in such circumstances, they responded to secondary infection. Lower function of lymphocytes and atrophy of lymph organs was found in animals infected with the field strain. These changes were transient and dependent of the size of the inoculum and prolonged shedding of *Salmonella*. However, the authors did not find any differences between the immunological responses on secondary bacterial infection. The colonization of the animal intestine with the filed strain was higher in chickens that received the filed strain of *Salmonella* compared to the vaccine strain.



Graph 1. Comparasion between the titre values in experimental groups 1 and 2.

We examined whether mixed infection affects body gain. A significant statistical difference at the level of 95% in body mass was found in the last week of the experiment, between the group of chickens infected with *S. Enteritidis* and *C. jejuni* and the group of chickens infected only with *C. jejuni*. This finding, as the one aforementioned, can be attributed to salmonella infection. Graph 1. presents the mean value of antibody titers in blood samples in the first and second experimental group.

In spite of the widespread nature of infections caused by *Campylobacter*, the clinical symptoms and pathology changes caused by these bacteria are rather rare. Campylobacteriosis can be expected if an additional factor is present, which affects the immune system and its ability to cope with the infection. This observation is supported by our experiments and can provide an answer on the matter of body gain and antibody titer to *C. jejuni*. What are the primary factors that influence the development of clinical symptoms is to be discovered in the future. Because this disease can be an indicator of the flock immune status we can suspect that mixed infection with *Campylobacter* and other agents occurs if the clinical symptoms characteristic for *Campylobacter* appear.

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## ISPITIVANJE UTICAJA INFEKCIJE SALMONELAMA NA POJAVU KAMPILOBAKTERIOZE KOD PILIĆA U EKSPERIMENTALNIM USLOVIMA

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### SADRŽAJ

Značajno mesto u ishrani ljudi zauzimaju meso i jaja živine, kao i različiti proizvodi od mesa i jaja. Kontinuirana kontrola zdravstvenog statusa živine u lancu proizvodnje jednako je važna kao i kontrola njihovih proizvoda namenjenih za ishranu ljudi. Uzročnici poput kampilobakterija i salmonela široko su rasprostranjeni u prirodi. Uticaj ovih bakterija na zdravlje životinja zavisi od imunskog statusa jedinki i u većini slučajeva kod imunološki nekompromitovanih jedinki infekcija ovim bakterijama ne dovodi do kliničkih simptoma. Za razliku od životinja, kod ljudi ove bakterije dovode do ozbiljnih kliničkih simptoma sa pojavom morbiditeta u visokom procentu. Njihovo prisustvo u proizvodima od živine, predstavlja jedan od glavnih puteva prenošenja ovih patogena koji izazivaju bolesti ljudi putem hrane. U ovom radu, ispitivanje je imalo za cilj da se kod živine veštački inficirane salmonelom ispita njen uticaj na kliničko ispoljavanje kampilobakterioze. Sigurno je da infekcija živine salmonelom utiče na imunološki sistem dovodeći do njegovog iscrpljivanja i opterećenosti što će omogućiti kampilobakterijama da se umnože do one mere kada će se pojaviti klinički znaci i razviti kampilobakterioza. U eksperimentalnim uslovima ispitivanje je izvedeno na tri grupe od po 30 pilića. Prvoj grupi je peroralno inokulisana suspenzija terenskih izolata *Campylobacter jejuni* i *Salmonella enterica* serovar *Enteritidis*; drugoj grupi peroralno je inokulisana suspenzija terenskog izolata *Campylobacter jejuni* i trećoj grupi peroralno je inokulisana suspenzija terenskog izolata *Salmonella enterica* serovar *Enteritidis*.

Prisustvo *Campylobacter jejuni* i *Salmonella Enteritidis* u organizmu veštački inficiranih pilića potvrđeno je reizolacijom uzročnika, a identifikacija izolovanih bakterija vršena je na osnovu njihovih morfoloških, kulturelnih i biohemijskih osobina. Imunološki odgovor inficirane živine i titar specifičnih antitela, kao i kretanje humoralnog imunskog odgovora na antigene *Campylobacter jejuni* kod eksperimentalnih pilića, praćeno je primenom reakcije vezivanja komplementa. Kod pilića inficiranih sojem *Campylobacter jejuni* i *Salmonella Enteritidis* uočeni su klinički znaci bolesti. Titar specifičnih antitela na *C. jejuni* u serumu inficiranih pilića utvrđen je reakcijom vezivanja komplementa (RVK). Rezultati ispitivanja su ukazali da infekcija salmonelama utiče na imunološki sistem pilića i tako stvara uslove da kampilobakterije naruše zdravstveno stanje domaćina.

