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Identification of Salmonella *rods in tissues of hens treated with selected antibiotics*

Wykrywanie pałeczek *Salmonella* w tkankach kur leczonych wybranymi antybiotykami

SUMMARY

The purpose of the studies was to compare the effectiveness of the methods of detecting *Salmonella* rods in tissues and inner organs of poultry. The studies compared the degree of sensitivity and specificity of bacteriological tests and PCR technique in the conditions modified by the presence of antibiotics. The studies were conducted on two groups: hens infected in natural conditions and chickens infected experimentally and treated with antibiotics according to the recommendations. Sections of liver, spleen, heart, duodendum fragments with pancreas, sections of ceca with tonsils and content, breast and femoral muscles and additionally sections of ovary and oviduct were taken for bacteriological tests and PCR. The following antibiotics were used: enrofloxacin (Enrocin, Grodziskie Zakłady Farmaceutyczne), norfloxacin (Nortril, Bremmer Farma), tiamphenicol (Tirsan, Fatro) and florphenicol (Nuflor, Schering-Plough Animal Health). The conducted bacteriological tests of chickens experimentally infected and subjected to treatment showed a considerable percentage of salmonella carrier state despite the therapy. The highest number of salmonella isolations was obtained from the birds treated with florphenicol. Salmonella was isolated from appendix in case of chickens treated with enrofloxacin and norfloxacin, while from chickens treated with tiamphenicol it was isolated from duodendum with pancreas. As was shown by the authors' own studies, the application of antibiotics to experimentally infected chickens does not completely free the animals from salmonella. *Salmoenalla* rods were isolated from experimentally infected chickens in all therapeutic groups and in positive control. A comparison of the results of bacteriological tests and PCR in birds subjected to antibiotic treatment showed that PCR is characterized by higher sensitivity and – hence – usefulness in detecting the carrier state of *Salmonella* rods in poultry.

Key words: *Salmonella Enteridis,* enrofloxacine, norfloxacine, tiamphenicol, florphenicol, bacteriological tests, PCR tests

INTRODUCTION

Reduced dissemination into the environment is observed during *Salmonella* infection in adult poultry. Fast reproduction of *Salmonella* rods and pathogen excretion occurs in chickens with

relatively simple (poor) intestinal flora. It may lead to quick *Salmonella* spread within the flock often due to infected water or fodder. Nowadays the applied methods for salmonella pollution control in poultry-house environment are inadequate to a still growing problem. Requirements referring to the improvement of the hygienic conditions are expensive and antibiotics applied for treatment favor the increase and spread of antibiotics-resistance. Vaccination that becomes a more and more useful and accepted method for infection control seems to be an alternative. Colonization of chickens' digestive tract by *Salmonella* may be also reduced by preparations made of natural intestinal flora of adult hens. It is called the competitive exclusion and affects the chicken's intestinal flora for their whole lives. Low pH is a factor that reduces the digestive tract colonization by *Salmonella*. Acidification of food content by *Lactobacillus* sp. occurs in the crop where preliminary digestion (fodder softening) takes place. Acidity in a crop remains at a constant level of pH 4–5 as a result of fermentation processes. Secretion of HCl and further pH decrease of the content occurs in proventriculus. There is a small intestine in the distal part behind the gizzard, then ceca branched from large intestine and joining with it just over the cloaca. Facultative anaerobes such as *Lactobacillus* sp., *Streptococcus* sp. and *Escherichia coli* are present in relatively large numbers in whole intestines, but obligatory anaerobes can be found in large numbers only in ceca. It is reflected in the acidity of food content of particular tract fragments. High bacteria viability affects the colonization of digestive tract by *Salmonella* rods [Darwin and Miller 1999]. They reach ceca and cloaca despite low pH in crop and gizzard. A great number of *Salmonella* reaches the distal fragments of intestines probably as a result of the weakening the flow and re-absorption of liquids from large intestinal content. The only method for reducing the *Salmonella* infections is antibiotics therapy, and despite of the fact that it favors drug-resistance appearance, it is still applied and brings some positive effects. However, the possibility of *Salmonella* rods carrying in poultry should be monitored because of the consumer's health [De Giessen van *et al.* 1992, Khakhria *et al.* 1997, Nastasi *et al.* 1998].

THE AIM OF STUDY

The aim of study was to compare the efficiencies of *Salmonella* rods identification methods in tissues and internal organs of poultry infected under natural and experimental conditions after antibiotics therapy, as well as to compare the sensitivity and specificity of bacteriological assays and PCR analysis under conditions modified due to antibiotics presence.

MATERIAL AND METHODS

Experiments were carried out using two groups: hens infected under natural conditions and chickens experimentally infected. Twenty-five hatching hens (consumption eggs) of Isa Brown breed aged 52 weeks naturally infected were divided into five groups (5 birds each). Four groups were treated with antibiotics according to drug producer's recommendations; the remaining group of naturally infected birds was the positive control (naturally infected but not treated).

Twenty-five 3-week-old chickens were subjected to experimental infection. Birds were divided into five groups (5 birds each). Four groups were infected and treated with antibiotics according to producer's recommendations; one group was the positive control (infected and not treated).

Sections of liver, spleen, heart, duodenum fragments with pancreas, fragments of ceca with tonsils and content, breast and femoral muscles; and sections of ovary and oviduct additionally from hatching hens were taken and subjected to bacteriological and PCR technique assays.

The following antibiotics were applied in the experiments: enrofloxacin (Enrocin, Grodziskie Zakłady Farmaceutyczne), norfloxacin (Nortril, Bremmer Farma), tiamphenicol (Tirsan, Fatro), and florphenicol (Nuflor, Schering-Plough Animal Health).

Chickens were infected by introduction into the crop of 1 ml of *Salmonella Enteritidis* suspension of optical density approximately corresponding to standard $1500 \cdot 10^6$ according to McFarland's grade.

The suspension for chicken infection was achieved in accordance with the methodology given by Rzedzicki *et al.* [2001].

A strain of *Salmonella* Enteritidis was also used to control the positive PCR reaction. *Escherichia coli*, *Citrobacter freundii*, *Klebsiella oxytoca* and *Proteus mirabilis* strains were applied to evaluate the specificity of PCR technique examinations.

Bacteriological assay

Particular organ sections were weighed and sealed in Stomacher's plastic bags (Seward, Anglia), to which buffered peptone liquid was added (Oxoid) in 1:10 ratio (w/v), and then homogenized in Stomacher's apparatus (Lab System, Model 80, Seward, England). Bacteriological assay was carried out in accordance with the methodology given by Rzedzicki *et al.* [2001].

Inoculation onto solid medium with nutrient agar was made from colonies characteristic of *Salmonella* and incubated at 37°C for 24 hours.

The achieved pure bacterial cultures were examined for attachment to *Salmonella Enteritidis* serovar using diagnostic serum (Biomed, Cracow).

PCR analysis

Particular sections of organs were homogenized adding buffered peptone liquid (Oxoid) in 1:10 ratio in Stomacher's apparatus (Lab System, Model 80, Seward, England). Homogenized samples were incubated at 37°C for 20 hours. Bacterial DNA was extracted using Genomic DNA Prep Plus kit in accordance with the producer's procedure.

A set of InvA1 (GTGAAATTATCGCCACGTTCGGGCAA), and InvA2 AAAGGAACC) starters (DNA – Gdańsk II) were applied to amplify DNA fragment of species-specific *Salmonella* rods according to Rahn *et al.* [1992].

RESULTS AND DISCUSSION

The pattern in UV trans-illuminator, where DNA band of 284 base-pairs weight was visible in gel, was considered as a positive result of PCR reaction. Such a result was achieved at 70% of experimentally-infected and antibiotics-treated chickens. The percentage of carrying in particular groups ranged from 25% and 5% (Fig. 1).

Taking into account the internal organs and muscle tissue, positive results were achieved from ceca, duodenum with pancreas, liver and heart (Tab. 1).

In a group of chickens treated with enrofloxacin, *Salmonella* presence was found in duodenum with pancreas and ceca. The presence of genetic material characteristic of *Salmonella* rods was found in ceca in all chickens, but in 40% of them both in ceca and duodenum with pancreas and at 60% of birds only in ceca. A similar situation was observed in the group treated with norfloxacin: the nucleotide sequence specific for *Salmonella* was identified in duodenum with pancreas and ceca in 40% of birds and only in ceca in remaining 60% of chickens. In the group treated with florphenicol, positive results were achieved in samples taken from liver and heart in 20% of chickens; from liver, heart and ceca in 20%; other 40% of birds showed positive results only from liver. It should be underlined that amplification products characteristic of InvA1/InvA2 starter pairs isolated from liver and heart originated exclusively from chickens treated with florphenicol. Positive results were achieved only from ceca of 20% of birds treated with tiamphenicol.

High *Salmonella* carrying in chickens treated with enrofloxacin as compared to those from control group was observed. Genetic material specific for *Salmonella* was found in ceca of all birds from that group. In control group, the percentage of carrying in ceca was slightly lower (Tab. 1). High percentage of positive results achieved from ceca in reference to control group was also recorded in chickens treated with norfloxacin. In the group treated with florphenicol, nucleotide sequence characteristic of *Salmonella* was found mainly in liver, while the percentage of positive results achieved from liver was 20% in control group (Tab. 1). No amplification products characteristic of InvA1/InvA2 starter pairs in breast and femoral muscles were found in experimental groups. The percentage of carrying in muscle tissue of control group was high amounting to 40 and 80%, respectively.

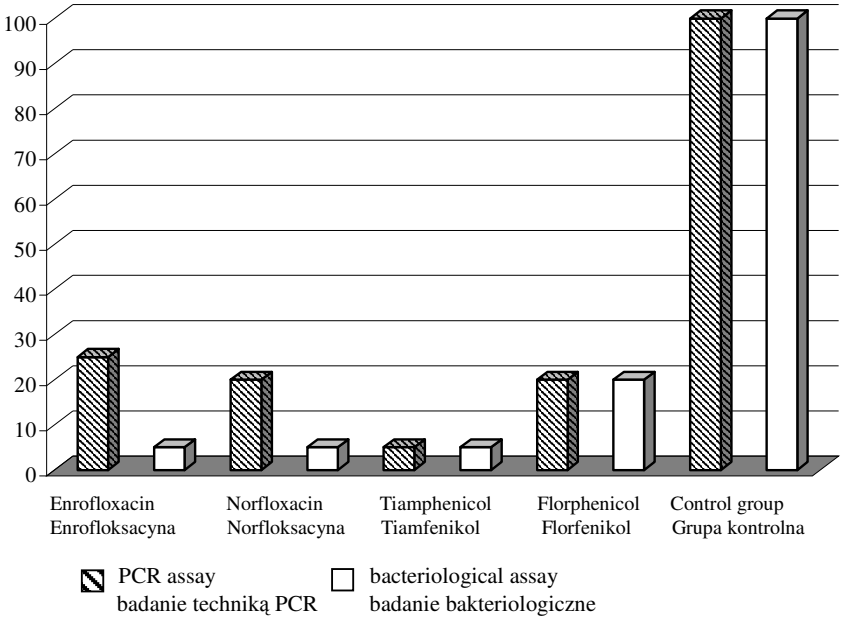

Fig. 1. Percentage of experimentally-infected and treated chickens with *Salmonella* presence
Rys. 1. Odsetek kurcząt zakażonych eksperymentalnie i poddanych leczeniu, u których wykryto obecność *Salmonelli*

Bacteriological assays of experimentally-infected and treated chickens revealed a significant percentage of *Salmonella* carrying despite the therapy. The highest number of *Salmonella* isolations was achieved in birds treated with florphenicol (Tab. 1) – the antibiotic recently applied for poultry disease therapy. *Salmonella* rods were isolated from liver and heart, but at the same time from both organs in 40% of chickens; only from liver in 20% and only from heart in 20% of birds. *Salmonella* was isolated from ceca in chickens treated with enrofloxacin plus norfloxacin, whereas from duodenum with pancreas in those treated with tiamphenicol. The comparison with control group results reveals that the percentage of all birds after antibiotics therapy being salmonella carriers was at a much lower level than in infected but not treated chickens. However, taking into account the carrying percentage in particular groups, high *Salmonella* isolation level in chickens treated with florphenicol as compared to the control

Table 1. Comparison of results achieved in bacteriological and PCR assays in experimentally-infected and antibiotics-treated chickens as well as control group with *Salmonella* presence

Tabela 1. Porównanie wyników uzyskanych w badaniu bakteriologicznym i techniką PCR w grupie kurcząt zakażonych eksperymentalnie i poddanych terapii antybiotykowej oraz kurcząt grupy kontrolnej, u których stwierdzono obecność *Salmonelli*

Groups Grupy	Organs and tissues – Organy i tkanki						
	Liver Wątroba	Serce Heart	Spleen Śledziona	Duodenum with pancreas Dwunastnica z trzustką	Ceca Jelito ślepe	Breast muscles Mięśnie piersiowe	Femoral muscles Mięśnie udowe
I [**]	0/0[*]	0/0	0/0	0/2 (40)	1/5(100)	0/0	0/0
II	0/0	0/0	0/0	0/2(40)	1(20)/4(80)	0/0	0/0
III	0/0	0/0	0/0	1(20)/1(20)	0/0	0/0	0/0
IV	4(80)/4(80)	2(40)/2(40)	0/0	0/0	0/1(20)	0/0	0/0
V	1(20)/1(20)	1(20)/1(20)	1(20)/1(20)	2(40)/2(40)	3(60)/3(60)	2(40)/2(40)	4(80)/4(80)

[*] bacteriological (%) / PCR (%) – badanie bakteriologiczne (%)/ badanie techniką PCR (%)

[**] group I – chickens treated with enrofloxacin
II – chickens treated with norfloxacin
III – chickens treated with tiamphenicol
IV – chickens treated with florphenicol
V – control

grupa I – kurczęta leczone enrofloksacyną
II – kurczęta leczone norfloksacyną
III – kurczęta leczone tiamfenikolem
IV – kurczęta leczone florfenikolem
V – kontrolna

group, was apparent. Referring to *Salmonella* rods presence in organs and muscles of control birds, *Salmonella* was isolated at the same time from heart and femoral muscles in 20% of birds in group; from liver, spleen and breast muscles in 20%, from duodenum with pancreas, ceca, breast and femoral muscles in 20%, and from ceca only in 20% of chickens. In that group, *Salmonella* rods were isolated from femoral and breast muscles in a significant percentage of chickens, while in *Salmonella*-treated groups, it was not isolated from muscles in all. Liver in chickens of therapeutic, as well as femoral muscles and digestive tract, namely ceca fragment, in control birds were predilectic site for *Salmonella* occurrence.

The autors' own study revealed that experimentally-infected chickens given antibiotics were not free from *Salmonella*. *Salmonella* rods were isolated from experimentally-infected birds in all therapeutic and positive control groups.

Salmonella rods were isolated in 35% of hens in a group naturally-infected and then treated with antibiotics. The highest isolation percentage was recorded among hens treated with florphenicol. In other treated groups, that percentage ranged between 10 and 5% (Fig. 2). Referring to *Salmonella* in hen's internal organs, it was isolated from liver, heart, spleen and ceca. In positive control group, *Salmonella* rods were isolated from all birds in femoral and breast muscles, oviduct, ovary, duodenum with pancreas, ceca, liver, heart and spleen (Tab. 2).

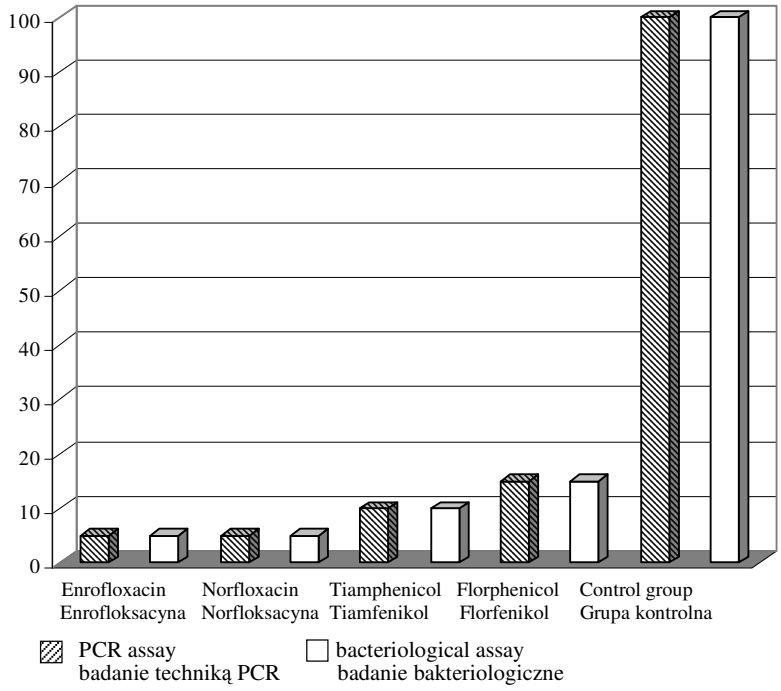

Fig. 2. Percentage of naturally-infected and treated hens with *Salmonella* presence
Rys. 2. Odsetek kur zakażonych w warunkach naturalnych i poddanych leczeniu, u których wykryto obecność salmonelli

Experiments revealed that *Salmonella* rods were isolated from spleen in hens treated with enrofloxacin like in those treated with norfloxacin. In a group treated with florphenicol, *Salmonella* was isolated from liver, heart and ceca; the one treated with tiamphenicol – from liver and ceca. *Salmonella* was isolated in birds from single internal organs (Tab. 2). The following percentage of birds had *Salmonella* isolated at the same time from many organs: control – duodenum with pancreas, breast and femoral muscles (20%), liver, duodenum with pancreas, ovary, oviduct and breast muscles (20%), heart, spleen, ceca, ovary, oviduct and breast muscles (20%), ovary, oviduct and breast muscles (20%), as well as duodenum with pancreas (20%). A comparison of the achieved results indicated a much higher percentage of *Salmonella* carrying in birds in control than therapeutic groups. The percentage of *Salmonella* rods isolation from internal organs was also at a much higher level. It is worth mentioning that in control group, *Salmonella* was isolated from ovary and oviduct and femoral and breast muscles, while in therapeutic groups results of bacteriological assays of these organs and tissues were negative.

Results achieved by to PCR technique application confirm bacteriological assays for naturally-infected hens and subjected to antibiotic treatment (Tab. 2).

A comparison of results for experimentally-infected and then treated chickens with those for naturally-infected and treated ones revealed a higher percentage of *Salmonella* isolations from particular organs of the former ones. In that group, *Salmonella* was isolated from two internal organs of florphenicol-treated chickens; in naturally-infected group – only from a single organ, thus the percentage of *Salmonella* rods isolation from both bird groups – chickens and hens – was at a similar level, despite the fact that the percentage of isolation from internal organs was higher in a group of experimentally-infected chickens.

The digestive tract, namely fragments of ceca and duodenum with pancreas, was a predilective site of *Salmonella* occurrence in chickens. PCR results for experimentally-infected chickens confirmed this observation. Literature data and the autors' own experiments results indicate that the digestive tract is extremely exposed to reproduction and living of *Salmonella* rods in an organism. The presence of *Salmonella*-specific nucleotide sequence was found in the digestive tract of every group of treated chickens. During bacteriological assay, *Salmonella* rods were isolated from the digestive tract of all groups except from that treated with florphenicol. Gast and Beard [1990] achieved the *Salmonella* isolation from ceca in 58% level, which was similar to that recorded here in control group of chickens. Zalesiński *et al.* [1990] found a slightly lower level of *Salmonella* occurrence in ceca.

A high percentage of *Salmonella* rods isolation from chicken's femoral muscles in control group is worth mentioning. Similarly, high level of carrying in chickens was observed in femoral muscle determined by means of PCR. Examination of breast muscles also revealed the presence of *Salmonella*. The percentage of positive results was at a lower level than in the case of femoral muscles, but in an aspect of human's health, the fact of isolation the *Salmonella* rods from muscles or their confirmation by PCR technique is alarming. No *Salmonella* occurrence in femoral and breast muscles of therapeutic groups was found either in bacteriological or PCR assays.

Studies upon the therapy using antibiotics against *Salmonella* rods mainly refer to quinolons application, enrofloxacin in particular. Its application in therapy appears to be highly effective, which favors fast disappearance of clinical symptoms. However, Humber *et al.* [1997] and Rzedzicki *et al.* [2001] found that *Salmonella* was not eliminated in all treated

Table 2. Comparison of results achieved in bacteriological and PCR assays in naturally-infected and antibiotics-treated hens as well as control group with *Salmonella* presence

Tabela 2. Porównanie wyników uzyskanych w badaniu bakteriologicznym i techniką PCR w grupie kur zakażonych w warunkach naturalnych i poddanych terapii antybiotykowej oraz kur grupy kontrolnej, u których stwierdzono obecność *Salmonelli*

Groups Grupy	Narządy i tkanki Organs and tissues								
	Liver Wątroba	Heart Serce	Spleen Śledziona	Duodenum with pancreas Dwunastnica z trzustką	Ceca Jelito ślepe	Ovary Jajnik	Oviduct Jajowód	Breast muscles Mięśnie piersiowe	Femoral muscles Mięśnie udowe
I **	0/0*	0/0	1(20)/1(20)	0/0	0/0	0/0	0/0	0/0	0/0
II	0/0	0/0	1(20)/1(20)	0/0	0/0	0/0	0/0	0/0	0/0
III	1(20)/1(20)	0/0	0/0	0/0	1(20)/1(20)	0/0	0/0	0/0	0/0
IV	1(20)/1(20)	1(20)/1(20)	0/0	0/0	1(20)/1(20)	0/0	0/0	0/0	0/0
V	1(20)/1(20)	1(20)/1(20)	1(20)/1(20)	3(60)/3(60)	1(20)/1(20)	3(60)/3(60)	3(60)/3(60)	4(80)/4(80)	1(20)/1(20)

* bacteriological (%)/PCR (%) – badanie bakteriologiczne (%)/badanie techniką PCR (%)

** group I – chickens treated with enrofloxacin — grupa I – kury leczone enrofloksacyną
group II– chickens treated with norfloxacin — grupa II – kury leczone norfloksacyną
group III– chickens treated with tiamphenicol — grupa III – kury leczone tiamfenikolem
group IV– chickens treated with florphenicol — grupa IV – kury leczone florfenikolem
group V– control group — grupa V – kontrolna

birds and *Salmonella* rods were isolated from quite a large percent of them after the antibiotic treatment. Similarly, in the autors' own study: *Salmonella* was isolated from a large percentage of chickens after the treatment using quinolons. The fact that isolation was made from the digestive tract, which favors the bacteria carrying into the environment, remains alarming.

The performed experiments revealed that the highest percentage of *Salmonella* isolations was achieved from hens treated with florphenicol. In groups treated with enrofloxacin and norfloxacin, the percentage was slightly lower. As compared to experimentally-infected chickens, for which *Salmonella* rods were isolated from ceca, the bacteria were isolated mainly from spleen in naturally-infected hens.

A comparison of bacteriological and PCR assays for birds subjected to antibiotic therapy revealed that PCR technique was characterized by higher sensitivity, and thus more suitability for identification of *Salmonella* rods carrying in poultry.

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STRESZCZENIE

Celem badań było porównanie efektywności metod wykrywania pałeczek *Salmonella* w tkankach i narządach wewnętrznych drobiu. Porównano stopień czułości i swoistości badań bakteriologicznych i badań techniką PCR w warunkach zmodyfikowanych obecnością antybiotyków. Badania przeprowadzono na dwóch grupach: kurach zakażonych w warunkach naturalnych

i kurczętach zakażonych eksperymentalnie leczonych antybiotykami zgodnie z zaleceniami. Do badań bakteriologicznych i badań techniką PCR pobierano wycinki wątroby, śledziony, serca, odcinka dwunastnicy z trzustką, odcinka jelit ślepych wraz z migdałkami i treścią, wycinki mięśni piersiowych i udowych, od niosek dodatkowo wycinki jajnika i jajowodu. W doświadczeniu wykorzystano następujące antybiotyki: enrofloksacynę (Enrocin, Grodziskie Zakłady Farmaceutyczne), norfloksacynę (Nortril, Bremmer Farma), tiamfenikol (Tirsan, Fatro), florfenikol (Nuflor, Schering-Plough Animal Health). Przeprowadzone badania bakteriologiczne kurcząt zakażonych eksperymentalnie i poddanych leczeniu wykazały znaczny odsetek nosicielstwa salmonelli, mimo przeprowadzonej terapii. Największą liczbę izolacji salmonelli uzyskano od ptaków leczonych florfenikolem. Od kurcząt leczonych enrofloksacyną i norfloksacyną salmonellę izolowano z jelita ślepego, natomiast od kurcząt leczonych tiamfenikolem – z dwunastnicy z trzustką. Jak wykazały prowadzone badania własne podawanie antybiotyków kurczętom zakażonym eksperymentalnie nie uwalnia całkowicie stada od salmonelli. Pałeczki *Salmonella* izolowano bowiem od kurcząt zakażonych eksperymentalnie we wszystkich grupach terapeutycznych oraz w grupie kontrolnej dodatniej. Porównanie wyników badań bakteriologicznych i techniką PCR uzyskanych od ptaków poddanych antybiotykoterapii wykazało, iż większą czułością, a co za tym idzie większą przydatnością w wykrywaniu nosicielstwa pałeczek *Salmonella* u drobiu, cechuje technika PCR.

Słowa kluczowe: *Sallmonella Enteridis*, enrofloksacyna, norfloksacyna, tiamfenikol, badania bakteriologiczne, badania techniką PCR