Praca oryginalna *Original paper*

Influence of the therapy of laying hens with selected antibiotics on the presence of Salmonella Enteritidis in the contents of the eggs

AGNIESZKA KOLASA, JERZY RZEDZICKI, MONIKA SKOWRON

Department Diseases of Birds, Institute of Biological Fundamentals of Animal Diseases, Faculty of Veterinary Medicine, University of Agriculture in Lublin, Głęboka 30, 20-612 Lublin

Kolasa A., Rzedzicki J., Skowron M.

Influence of the therapy of laying hens with selected antibiotics on the presence of Salmonella Enteritidis in the contents of the eggs

Summary

One of the methods that can reduce the transmission of Salmonella from the reproductive tract to eggs can be the application of antibiotic therapy of infected hens. Studies characterizing the antibiotics effectiveness in salmonellosis therapy have shown that the therapy can only decrease the number of the bacterial population but it does not fully eliminate bacteria. The aim of the study was the assessment of the therapy of hens with selected antibiotics on the presence Salmonella Enteritidis in the content of eggs. The investigations were conducted in two stages. In the first stage the hens were infected experimentally with Salmonella Enteritidis. In the second stage the hens that had been infected were treated with selected antibiotics (enrofloxacine, norfloxacin, flumequine, amoxycillin and amoxicillin-clavulanic acid). In the present study S. Enteritidis was isolated from eggs that were laid during a 12 to 24 day interval post inoculation in all experimental groups but at individual days the quantity of infected eggs varied. On the 24th day post inoculation there was the highest amount (29.4%) of all contaminated eggs. After antibiotic therapy in some groups of hens there were no infected eggs. This pertained to the birds which were treated with enrofloxacine and norfloxacin. The result of the therapy with amoxycilln, amoxicillin-clavulanic acid and flumequine in the other groups was only the reduction of the quantity of infected eggs. The findings presented above demonstrate that the antibiotic therapy of hens infected with Salmonella Enteritidis has not always been effective in the elimination of these bacteria from the tissues of the reproductive tract but the therapy with enrofloxacine and norfloxacin can eliminate the possibility of salmonellas transmission into the contents of the eggs. Moreover, these examinations have shown the correlation between the infection of reproductive tract organs (ovary, oviduct) and the contents of eggs (yolk, white) that were laid by hens infected with Salmonella Enteritidis.

Keywords: Salmonella, eggs, antibiotics

The ubiquitous presence in the environment of many *Salmonella* serovars, not strictly adapted to one definite host, e.g. *Salmonella* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Virchow, can explain resultant human and animal infections. There are a great number of *Salmonella* serovars which can be isolated from poultry. Most of them do not induce clinical symptoms in infected birds, but simultaneously they are responsible for significant percentage of infections in consumers of poultry products. Among them are: *S.* Senftenberg, *S.* Montevideo, *S.* Agona, *S.* Bredeney.

Eggs can be infected both in the body of laying hens as well as in the environment. Despite the presence of the egg shell covered by a cuticule and the egg shell's membranes constituting a mechanical barrier, microorganisms can penetrate inside the egg both through the pores and micro-damages of egg shell structure. The dynamics of *Salmonella* multiplication inside the egg content depends on temperature and conditions of eggs' storage (6, 15). One of the methods which can reduce transmission of *Salmonella* from the reproductive tract of infected hens to eggs is antibiotic therapy. Studies on the effectiveness of antibiotics in salmonellosis therapy have shown that the therapy can only decrease the number of bacteria but it cannot fully eliminate salmonellae.

Most of the drugs bind to serum proteins, especially to albumins, α1-acid glycoproteine, and to tissues proteins (13). The drug residue in tissues is gradually eliminated after the end of drug administration. Thus, there is a problem with a minimal residue of antibiotics in tissues and eggs irrespectively of the method of

their application. These situations should be considered as undesirable. The residues of antibiotics in food are harmful not only for the human body but they also restrain bacterial growth and hence they may affect the result of bacteriological examinations of food products (4, 10).

The aim of the study was the assessment of the antibiotic therapy of infected hens on the presence *Salmonella* Enteritidis in egg contents.

Material and methods

35 Isa Brown hens, 53-weeks-old, were examined. All the birds were accommodated in individual cages. Pelleted feed and tap water were provided *ad libitum.* The examinations were conducted in two stages. In the first stage the hens were infected experimentally with *Salmonella enterica* subsp. *enterica* serovar Enteritidis strain. Isolation and identification of this strain was carried out by the Department of Bird Diseases in Lublin. The strain was isolated from hens from the Lublin district. The experimental infection was conducted by the application of 1 ml *Salmonella* Enteritidis suspension directly to the crop of each hen. The density of *Salmonella* Enteritidis suspension was estimated as 1500×10^6 according to the McFarland scale. The experimental infection of hens was conducted through three consecutive days. The cloacal swabs, serum and eggs were chosen for examinations.

In the second stage the infected hens were treated with selected antibiotics. The eggs and internal organs were taken for examinations.

The hens were divided into seven groups (five hens in each group). Five groups out of seven were infected birds that were treated (according to instruction of drug producers). The other two groups served as a negative control (K– no infected hens) and a positive control (K+ no treated infected hens).

Cloacal swabs were taken from each bird and assigned for bacteriological examination. The samples were taken before infection (sample 0) and on 7th day after infection of birds.

1.5 ml of blood from the *vena ulnaris* was taken from each bird and serologically examined. The samples were taken before infection (sample 0) and on the 14th and 21st days after infection of the birds.

The collection of eggs started on the following day after the experimental infection of hens and it lasted for 24 days after experimental infection. The hens which were divided into five groups were then exposed to five or seven days of antibiotic therapy. The eggs were collected again the next day after the antibiotic therapy had been finished. The eggs were collected daily and stored at 7°C.

The samples of ovaries, oviducts and spleens were taken from birds after euthanasia and assigned to bacteriological examination.

The following antibiotics were used: enrofloxacine (Enrocin Grodziskie Zakłady Farmaceutyczne), norfloxacin (Nortril Bremmer Farma), flumequine (Bioflumeq, Vetoquinol Biowet), amoxycillin (Biomox, Vetoquinol Biowet) and amoxicillin-clavulanic acid (Amoksiklav, Lek Polska). The scheme of the experiment is shown in tab. 1.

Tab. 1. The scheme of experiment

Group	Experimental infection	Antibiotics
K–	–	no
K+	+	no
I	+	enrofloxacine
II	+	norfloxacin
III	+	flumequine
IV	+	amoxicillin
V	+	amoxicillin-clavulanic acid

Antibiotic susceptibility of *Salmonella* Enteritidis strain was performed on the Mueller-Hinton medium (BTL) by antimicrobial disc test against the following contents of antibiotics: enrofloxacine (ENR – 5 µg), norfloxacin (NOR – 10 µg), flumequine (UB – 30 µg), amoxicillin (AML – 25 µg), amoxicillin-clavulanic acid (AMC – 30 µg). Susceptibility and resistance were delineated using the breakpoint and zones size criteria set by NCCLS (1, 8).

Preparation of egg yolk and albumen for examinations. Eggs shell surfaces were disinfected by dipping in 70% ethanol for 5 sec, and the shells were then broken and yolk and albumen separated against sterile foil strips.

Preparation of samples of internal organs. Individual samples of ovary, oviduct and spleen were weighed and then placed into sterile foil strips. The samples were mixed in the ratio of 1 to 10 with Buffered Peptone Water (Oxoid) and then homogenized by Stomacher (Lab System, Model 80, Seward, England).

Bacteriological examination. Each of the samples was inoculated in Buffered Peptone Water (Oxoid) supplemented with 35 mg/L ferrous sulfate and incubated for 20 hr at 37°C. A 1-ml portion from each incubated BPW broth culture was transferred to both Rappaport-Vasiliadis broth (RV) (Oxoid) and Muller-Kauffmanns' broth (MK, Oxoid) and incubated for 48 hr at 42°C with shaking. An inoculating loop was used to streak each incubated RV and MK broths culture onto brilliant green agar (BGA, Oxoid) and onto xylose lysine sodium deoxycholate (XLD) agar (Oxoid). Then the agar plates were incubated at 37°C for 24-48 hr.

The identity of *S.* Enteritidis colonies was confirmed biochemically (API 20E, bioMérieux) and serologically.

Examination of the content of the eggs and internal organs by PCR technique. The samples of eggs and internal organs that were determined as infected by the bacteriological examination were examined by the polymerase chain reaction technique. Bacterial DNA was extracted from examined samples using enrichment in BPW. Primers were selected on the basis of criteria described by Rahn et al. (14). A pair of primer sequences designed as InvA1 (GTGAAATTATCGCCACGTTCGGGCAA) and InvA2 (TCATCGCACCGTC AAAGGAACC). The primers were synthesized by „DNA – Gdańsk II." Thermal cycling was conducted with the following conditions: an initial denaturation cycle at 94°C for 3 min., denaturation at 95°C for 5 s, annealing at 60°C for 30 s, extension at 72°C – 40 s (last cycle 3 min.), and the number of cycles – 40. A 10 µl portion of the sample after PCR was analyzed by electro-

phoresis in agarose gel (1.5%), stained by ethidium bromide (1 µg/ml) and visualized in UV-light illumination. A DNA molecular weight standard n. 100 bp (GeneRuler™, DNA Ladder Plus, Fermentas) was analyzed along with the samples. The presence of a DNA fragment of 284 bp was recognized as a positive result. Specificity of PCR reaction was performed with one *Salmonella* Enteritidis and one *Salmonella* Typhimurium strain as a positive control along with other bacteria that belong to *Enterobacteriaceae* (*Escherichia coli*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Proteus mirabilis*) as a negative control.

Serological examination. Serum samples, collected before inoculation and at 14 and 21 days thereafter, were tested for the presence of specific anti-*Salmonella* antibodies by the commercial enzyme-linked immunosorbent assay (ELISA) (Flock Check SE, IDEXX). Color development was evaluated at 650 nm in a Labsystems Multiscan Plus reader. The magnitude of the antibody response associated with each serum sample was estimated as a ratio of the sample ELISA absorbance value to the mean absorbance value of the negative control samples.

The correlation coefficient between infection of reproductive tract organs (ovary, oviduct) of hens and the contents of eggs (yolk, white) which were laid by these hens was evaluated by the Statistica 6.0. (Kołmogorowa test). The obtained data was presented as a correlation coefficient (r)*, which value was higher as the relationship between two variables.

Results and discussion

The bacteriological examination of cloacal swabs from hens before experimental infection did not show *Salmonella* Enteritidis infection. *Salmonella* Enteritidis was isolated from 91.6% of the samples that were taken on the 7th day after infection. None of the samples from the negative control group (K–) was infected by *Salmonella*. No hens were detected as seropositive before inoculation with *S.* Enteritidis. The level of antibodies increased visibly on the 14th day post inoculation. The value of S/N on this day varied between 0.620 and 0.338. At the 21st day after inoculation the levels of anti-*Salmonella* antibodies were included within the positive results limit in all examined birds. Detailed results of serological examination are shown in tab. 2.

The strain of *Salmonella* Enteritids that was used in the examinations was fully susceptible to the applied antibiotics.

During the sampling interval from 3 to 24 days post inoculation, *S.* Enteritidis was recovered from 8.9% of the eggs. The total amount of eggs that were laid in this interval was 205. This result was very similar to that reported by Humphrey et al. (7), in which they examined artificially infected with *Salmonella* Enteritidis hens and they found 7.4% of the eggs infected by *Salmonella*. Compared with that result, the same

Tab. 2. The magnitude of the antibody response before and after experimental inoculation of birds in all groups (n = 5; median of S/N coefficient ± s)

Group	Days after inoculation of birds					
	0		14		21	
K–	1.308	0.105	1.257	0.115	1.216	0.083
K+	1.451	0.102	0.453	0.090	0.854	0.238
I	1.207	0.098	0.409	0.247	0.298	0.120
II	1.180	0.102	0.408	0.233	0.254	0.202
III	1.372	0.143	0.620	0.208	0.376	0.225
IV	1.197	0.141	0.338	0.144	0.373	0.095
V	1.406	0.167	0.500	0.244	0.408	0.196

authors (6) isolated from 0.9% to 1.1% infected eggs in two naturally infected flocks.

In the present study *S.* Enteritidis was isolated from eggs that were laid from a 12 to 24 day interval post inoculation in all experimental groups, but at individual days the number of infected eggs varied. On the 24th day post inoculation there was the highest amount (29.4%) of all contaminated eggs.

After antibiotic therapy of the hens in some groups there were no infected eggs. This pertained to the birds which were treated with enrofloxacine and norfloxacin. Therapy with amoxycilln, amoxicillin-clavulanic acid and flumequine in the other groups only decreased the number of infected eggs. Presumably a possible influence on this effect can be good absorption and biologic availability of fluoroquinolones after oral administration of these therapeutics (2). Moreover, fluoroquinolones can enter cells easily, reaching maximum concentration in a short period after administration, and therefore they are often used to treat intracellular pathogens.

Salmonella Enteritidis bacteria might be isolated from the mucosal epithelim surface as well as within epithelial cells of the oviduct (5). Tubular glands of the isthmus are the predominant colonization site of *Salmonella* Enteritidis in the upper oviduct of laying hens (3). Lower efficacy of the therapy that was applied in other groups could be caused by intracellular infection with *Salmonella* Enteritidis. In these groups, despite the application of antibiotic therapy *Salmonella* Enteritidis was still isolated from the eggs but the total number of infected eggs was lower. The number of infected eggs was insignificantly lower after amoxicillin administration. After administration of amoxicillin-clavulanic acid and flumequine the number of infected eggs were significantly lower. The percentage of infected eggs with *Salmonella* Enteritidis before and after antibiotic therapy in individual groups is shown on fig. 1.

The influence of antibiotics therapy on the natural defensive mechanisms of egg presents a different problem. To some extent, the antibiotic therapy can exert an influence on the efficiency of natural antibacterial

* Interpretation: r = 0 variables are not correlated; r < 0.1 faint correlation; r ≤ 0.3 weak correlation; r < 0.5 mediocre correlation; r < 0.7 high correlation; r ≤ 0.9 very high correlation or almost complete correlation, r = 1 maximum correlation; r = –1 negative correlation.

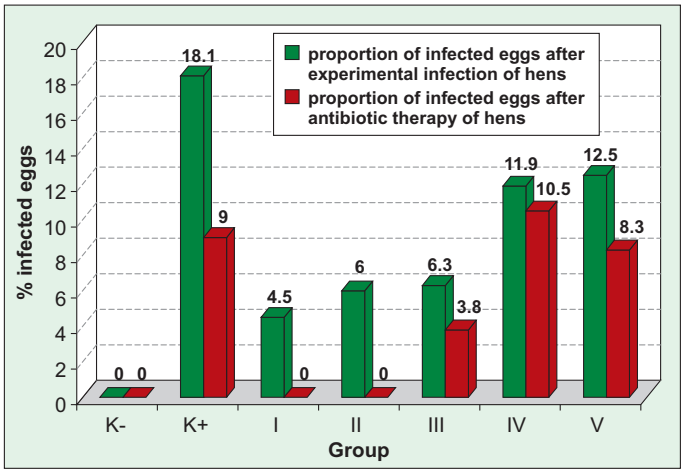

Fig. 1. The percentage of infected eggs with *Salmonella* Enteritidis before and after antibiotic therapy in individual groups

substances which are present inside egg contents. These may be a result of the reduction of lysozyme and ovomucin activity or other protein fractions and, therefore, a change of white egg pH and disorder its gel structure. These not fully known changes can indirectly disorder the natural defensive function of egg (16).

It is presumed that in the course of infection *Salmonella* Enteritidis possesses an ability to invade the tissues of the chicken reproductive tract (3, 9, 11). According to Okamura et al. (12) there is a correlation between the frequencies of egg yolk or egg white infection by *Salmonella* and infection of chicken reproductive tract organs (ovary and/or oviduct).

In the groups of experimentally infected hens, *Salmonella* was isolated from the reproductive organs of 31.4% of the birds. The percentage of infected organs in individual groups is shown in tab. 3.

The highest proportion of infected ovaries occurred in two groups in which the hens were treated with amoxicilline and flumequine. In the group of hens which were treated with amoxicillin there was a high correlation (r = 0.9) between the quantities of infected ovaries and egg yolks laid by these hens. Distinct from that, the mediocre correlation degree (r = 0.6) between infected ovaries and egg yolks was noticed in the positive control group (K+). There was no such correlation in the group of hens which were treated by flumequine although there were 40% of infected ovaries. The correlation between infected oviducts and egg whites was observed in three out of all the groups. The maximum coefficient of correlation (r = 1) between infected oviducts and egg whites occurred in the group of birds which were treated with amoxicillin, however in the group of hens which were treated with amoxicillin clavulanic acid and fumequine the correlation was mediocre (r = 0.6).

In conclusion, the findings presented above have shown that the antibiotic therapy of hens infected with *Salmonella* Enteritidis has not been always effective in the elimination of these bacteria from the tissues of

Tab. 3. Percentage of infected organs in individual groups of examined birds

Group	Proportion of infected organs		
	% ovary	% oviduct	% spleen
K–	0	0	0
K+	40	20	0
I	0	20	0
II	0	20	0
III	40	40	0
IV	40	20	20
V	20	20	0

the reproductive tract but the therapy with enrofloxacine and norfloxacin can eliminate the possibility of salmonellae transmission into the content of the eggs. Moreover, these examinations have shown the existence of a correlation between infection of the reproductive tract organs (ovary, oviduct) and the content of eggs (yolk, white) which were laid by hens infected with *Salmonella* Enteritidis.

References

1. *Anon.*: National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals – Second Edition: Approved Standard M31-A2. NCCLS, Wayne. PA., USA 2002.
2. *Chylak J.*: Skuteczność i farmakokinetyka wybranych chinolonów. Pol. Tyg. Lek. 1993, 48, 860-863.
3. *De Buck J., Pasmans F., Van Immerseel F., Haesebrouck F., Ducatelle R.*: Tubular glands of the isthmus are the predominant colonization site of Salmonella Enteritidis in the upper oviduct of laying hens. Poult. Sci. 2004, 83, 325-358.
4. *Gustafson R. H., Bowen R. E.*: Antibiotic use in animal agriculture. J. Appl. Microbiol. 1997, 83, 531-541.
5. *Hoop R. K., Pospischil A.*: Bacteriological, serological, histological and immunohistochemical findings in laying hens with naturally acquired Salmonella enteritidis phage type 4 infection. Vet. Rec. 1993, 133, 391-393.
6. *Humphrey T. J., Baskerville A., Mawer S., Rowe B., Hopper S.*: Salmonella enteritidis phage type 4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol. Infect. 1989, 103, 415-423.
7. *Humphrey T. J., Cruickshank J. G., Rowe B.*: Salmonella enteritidis phage type 4 and hens' eggs. Lancet i 1989, 281.
8. *Kałużewski S.*: Zasady i metodyka oznaczania wrażliwości bakterii na chemioterapeutyki w rutynowych badaniach diagnostycznych. PZH, Warszawa 1976.
9. *Keller L. H., Benson C. E., Krotec K., Eckroade R. J.*: Salmonella Enteritidis colonization of the reproductive tract and forming and freshly laid eggs. Infect. Immun. 1995, 63, 2443-2449.
10. *Memish Z. A., Venkatesh S., Shibl A. M.*: Impact of travel on international spread of antimicrobial resistance. Internat. J. Antimicrob. Agents 2003, 21, 135-142.
11. *Miyamoto T., Baba E., Tanaka T., Sasai K., Fukata T., Arakawa A.*: Salmonella Enteritidis contamination of eggs from hens inoculated by vaginal, cloacal and intravenous routes. Avian Dis. 1997, 41, 296-303.
12. *Okamura M., Kamijima Y., Miyamoto T., Tani H., Sasai K., Baba E.*: Differences among six Salmonella serovars in abilities to colonize reproductive organs and contaminate eggs in laying hens. Avian Dis. 2001, 45, 61-69.
13. *Pacifici G. M., Viani A.*: Methods of determining plasma and tissue binding of drugs: pharmacokinetic consequences. Clin. Pharmacokinet. 1992, 23, 449-468.
14. *Rahn K., De Grandis S. A., Clarke R. C., McEven S. A., Galan J. E., Ginocchio C., Curtiss R. 3rd, Gyles C. L.*: Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. Mol. Cell Probes 1992, 6, 271-279.
15. *Timoney J. F., Shivaprasad H. L., Baker R. C., Rowe B.*: Egg transmission after infection of hens with Salmonella Enteritidis phage type 4. Vet. Rec. 1989, 125, 600-601.
16. *Trziszka T.*: Odchylenia jakościowe w surowcu jajczarskim wywołane kontaminacją pasz mikotoksynami oraz wpływem kokcydiostatyków i antybiotyków. Medycyna Wet. 1994, 50, 26-29.

Adres autora: dr Agnieszka Kolasa, ul. Głęboka 30, 20-612 Lublin; e-mail: agnieszka.kolasa@ar.lublin.pl