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Research article

Impact of rearing system of Rosa breed laying hens on occurrence and antibiotic resistance of *Salmonella* spp. isolated from egg shell surface

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Abstract: *Salmonelloses are among the most common animal-borne infections. The most frequent causes of their occurrence are contaminated chicken eggs. For this reason, the control of bacteria from the Salmonella genus living in the cloaca of laying hens can contribute to the reduction of spreading levels of infections with these bacteria. The most popular methods of rearing laying hens in small agricultural farms comprise run and cage keeping. The aim of the performed studies was to determine the impact of rearing and nutritional systems on the occurrence of egg infections with Salmonella spp. bacteria. Detection by PCR method corroborated presence of bacteria from the Salmonella genus on eggs surface from hens kept in litter system. Latex serotyping test confirmed the presence of Salmonella Enteritidis. Salmonella spp. was not detected on eggs surface from hens kept in cage system. Salmonella spp. isolates from the eggs surface of hens reared on litter were characterized by drug resistance to tetracycline. Addition of EM probiotic failed to show reduction in incidence of Salmonella spp. infection.*

Keywords**:** *Salmonella, laying hens, antibiotic resistance, rearing system.*

Introduction

Salmonelloses are one of the most frequent causes of animal-borne infections. They are caused by Gram negative, intestinal bacteria – *Salmonella* spp. which belong to the family of *Enterobacteriaceae*. It was demonstrated that majority of *Salmonella* spp. serotypes causing diseases in people can be transferred through infected stocks of poultry, meat, milk and their products [1, 2]. Poultry is an important source of diseases of the gastrointestinal tract most frequently associated with the consumption of infected hen eggs often consumed

without prior thermal treatment which is a key moment reducing pathogens [3]. The presence on a farm of rods of *Salmonella* spp. genus may be a cause of eggshell contamination with this pathogen. Reduction of *Salmonella* spp. bacteria populations living in the cloaca of hens can reduce infections with these bacteria [4]. Monitoring of the frequency occurrence with *Salmonella* spp. infections carried out since 2005 indicates that the main risk factor associated with infections is connected with hygiene conditions and, to a certain extent, also with the employed rearing system [5]. In the case of pathogenic bacteria, their drug resistance constitutes an important problem for antibiotic therapy. At the present time, manufacturers often apply probiotic additives to feeds as an alternative to antibiotics [6] and also to eliminate transfer of undesirable microflora between hens in the stock [7]. Moreover, sometimes these additives are also used as factors of pathogen internal control [8].

The objective of the performed investigations was to compare the effect of the litter and cage rearing systems on levels of egg infection with *Salmonella* spp. and their drug resistance.

Experimental

Materials

Eggs for experiments were obtained from experimental farm situated in Wielkopolska Voivodeship and derived from Rosa (40 weeks of age) breed laying hens which were under veterinary care (free from *Salmonella* spp.). Eggs were derived from Rosa hens reared on litter (n = 40) and cage (n = 40) systems (both covered with chopped straw). Breeding in cages and litter according to Council of Europe recomendations [9].

Each stock was fed diets (free from *Salmonella* spp.) containing: soybean meal (25%), maize (21%), wheat (43%), plant oil (1.4%), monocalcium phosphate (0.6%), chalk (8%), Lutamix (1%), metabolic energy (11.5) MJ, crude protein (18.5%).

During the first week of the experiment 240 eggs were collected (151 eggs from hens reared on cage system, and 89 eggs from hens reared on litter system).

Methods

The experiments comprised fresh, unwashed, collected eggs from each experimental combination. Bacteria from eggshells were rinsed in 200 ml solution of physiological salt at 37^{o}C for 60 minutes. The eluted bacteria were centrifuged (5000 rpm; 10 min) and the sediment was suspended in physiological salt to prepare a number of decimal dilutions. Next, 200 µl suspension was collected from the obtained dilutions and transferred onto a plate with XLT4 agar (Xylose-Lysine-Tergitol4 – Merck), in order to identify *Salmonella* spp. The incubation was conducted for 18-24 hours at the temperature of 37^{o}C. Following the procedure recommended by the manufacturer (Bacterial genomic DNA

– Sigma kit), DNA was isolated from the cultured colonies and then the amplification reaction was performed using the PCR method. Starters specific for the *inv* A gene of *Salmonella* spp.: Sal-F 5' TAT CGC CAC GTT CGG GCA A 3', Sal-R 5' TCG CAC CGT CAA AGG AAC C 3' which yield products of the size of 275 bp were applied. The volume of the reaction mixture was 25 µl. The reaction mixture consisted of 25 mM dNTP, 5 pm of each starter, 0.2 U polymerase Taq, 1 µl bacterial DNA. The amplification conditions were as follows: Amplification conditions: 94°C for 15s, then 35 cycles: 94°C for 3s, 50°C for 10 s,74°C for 35s, final extension 74°C for 2 min. The PCR product image was observed on 2% agarose gel with ethidium bromide (10 mg ml^{-1}). Serological identification of strains was performed using *Salmonella* Latex Test Kit (Biomed).

In the course of investigations, drug resistance of the isolated bacteria to the following selected antibiotics was determined: chloramphenicol (30 µg), gentamicin (10 µg), tobramycin (10 µg), and tetracycline (30 µg).

Drug resistance of the bacteria of the *Salmonella* spp. genus determined on the basis of the PCR product was checked using the Kirby-Bauer diffusion-disc method. Sterile discs (bioMerieux) saturated with antibiotic were placed on the Mueller-Hinton agar (Difco) on the lawn with the suspension of the tested microorganism adjusted to 0.5 on the McFarland scale and incubated at the temperature of 37°C for 18 hours. The zone of inhibition area of bacterial growth around the antibiotic disc was interpreted in accordance with CLSI standard and the manufacturer's recommendations.

Laying hens kept in litter system were additionally given an EM probiotic supplied in water after detection of *Salmonella* spp. by PCR method. The composition of the applied EM probiotic was as follows: (*Lactobacillus casei*, *Lactobacillus plantarum* 5.0 x 10 CFU ml^{-1}, *Saccharomyces cerevisiae* 5.0 x 10 CFU ml^{-1}, *Rhodopseudomonas palustris*), molasses and water (composition according to the manufacturer's leaflet, Greenland Technology EM). The volume of 3 l of the probiotic was diluted in 1000 l water and supplied to experimental birds for the period of 30 days. Eggs for analyses were collected during the last week of duration of the experiment.

Results and discussion

Using XLT4 agar with tergitol, growth of a few bacterial colonies was observed (eggs derived from hens reared on litter). Not all of the colonies were characteristic for *Salmonella* spp. (according to the manufacturer's description). *Salmonella* spp. was not detected on eggs surface from hens kept in cage system. DNA isolated from each colony H_2S-positive black or black-centered with a yellow periphery was used as a template in the polymerase chain reaction. In 3 samples (3.4%) from surface of eggs derived from hens kept on litter a PCR product of 275 bp was obtained characteristic for the *inv*A gene fragment

of *Salmonella* spp. (Figure 1). Detection by latex serotyping showed the presence of *Salmonella* Enteritidis.

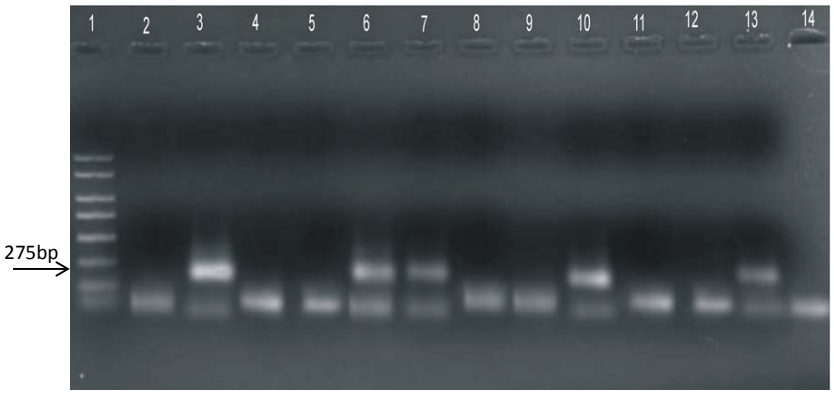

Figure 1. 2% agarose gel electrophoresis of PCR product (275 bp) amplified with primers Sal-F i Sal-R characteristic for *inv*A gene. Line 1 – Molecular weight marker, Line – 2-7 feed without probiotic, Line – 8-14 after probiotic application in feed

After 30 days of application of EM probiotic 89 eggs were taken and microbiological, PCR and latex analysis was carried out again. In 2 samples (2.2%) from surface of eggs derived from hens kept on litter a PCR product of 275 bp was obtained characteristic for the *inv*A gene fragment of *Salmonella* spp. Detection by latex serotyping confirmed the presence of *Salmonella* Enteritidis. Colonies of *Salmonella* Enteritidis were subjected to the test for drug resistance (Table 1).

Table 1. Drug resistance of *Salmonella* Enteritidis isolated from eggs of hens kept on litter

Drug resistance	**Chloramphenicol** 30 µg		**Gentamycin** 10 µg		**Tobramycin** 10 µg		**Tetracycline** 30 µg	
	K	P	K	P	K	P	K	P
	18	20	15	15	15	15	6	6
Resistance*	≤12		≤12		≤12		≤11	
Susceptibility*	≥18		≥15		≥15		≥15	

K – control – feed without probiotic; P – feed supplemented with probiotic; * CLSI [6]

As a Member State of the European Union since 2004, Poland is obliged to obey appropriate directives concerning the control of *Salmonella* spp. as well as other pathogenic factors transmitted by food. From that date, in the case of breeding stocks of hens (*Gallus gallus*), large stocks of these birds are strictly monitored in an attempt to restrict the frequency of occurrence of *Salmonella* spp. serotypes [5]. In April 2012, the Council of Ministers issued an attachment for

years 2012 and 2013 regarding domestic program of control of some serotypes of *Salmonella* spp. in breeding stocks of the hen (*Gallus gallus*) species. In this study, an attempt was made to analyse the occurrence of *Salmonella* spp. bacteria on the shell surface of eggs derived from hens from different rearing systems and, additionally after positive identification on XLT4 agar and by PCR method, fed an EM probiotic and to assess the occurrence of drug resistance in *Salmonella* spp. to selected antibiotics. Conventional microbiological methods applying selective medium substrates failed to provide unequivocal results. Such result could have been caused by sample microbiological contamination making it difficult to identify bacteria from the *Salmonella* spp. genus. Isolation of the DNA material from the developed colonies and the performed PCR reaction using starters specific for the *inv*A gene fragment of *Salmonella* spp. made it possible to identify isolates containing the sought bacteria. The Kirby –Bauer Disc Diffusion Test performed on the Mueler-Hinton agar medium have shown that the analysed bacteria are resistant to tetracycline. This kind of resistance is not surprising bearing in mind the fact that this antibiotic was frequently used in the past as a growth stimulator in animals.

Hen eggs may become contaminated in many ways, among others: parental stocks, hatcheries, environment, feeds, litter as well as water and, therefore, it is understandable that the presence of organic compounds on the surface of eggshells increases probability of survival of *Salmonella* spp. [10]. These factors contribute to the occurrence of both primary and secondary infections. For these reasons, it is essential for breeders who have both big and small stocks of hens but also farmers who keep laying hens for domestic use to follow strictly all hygiene procedures and undertake appropriate measures as soon as they discover a focus of the disease. Infection hazards of hen stocks with *Salmonella* spp. are also associated with such factors as the size of the stock, age of layers as well as the employed rearing system [3]. In our experiments, no bacteria which were the object of investigations were found on the surface of eggs derived from hens kept in cages, whereas in the case of eggs derived from hens kept on litter, 3 isolates characterised as *Salmonella* spp. on the basis of the performed PCR reaction were determined. Housing systems which include outdoor areas may be especially vulnerable to Salmonella introduction from external environmental sources [11]. The addition of probiotic, although it exerted a positive influence on poultry intestinal microflora, yet it failed to have a significant impact on the *Salmonella* spp. drug resistance. This constitutes a problem because there is a possibility of infections with *Salmonella* spp. by way of consumption of contaminated food articles of animal origin.

Among bacterial populations found on eggs surfaces of hens kept on litter, *Salmonella* spp. were determined with the assistance of the PCR method. *Salmonella* spp. bacteria isolated from the surface of eggs derived from hens kept on litter were resistant to tetracycline.

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