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STUDY OF THE PRESENCE OF SPECIFIC *SALMONELLA* ENTERITIDIS ANTIBODIES IN CHICKEN EGG YOLKS BY COMPETITIVE cELISA METHOD

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One of the most common causes of salmonellosis of man and poultry is Salmonella Enteritidis which is often found in the digestive system of adult birds. The infected birds do not display any evident clinical symptoms and, at the same time, they excrete the bacteria into the surrounding environment. Studies are carried out by standard microbiological procedures which include the isolation of Salmonella spp. in egg yolks and their serologic typization by agglutination on microplates. Along these methods, studies on the possibility to use an enzyme immunoassay, such as cELISA, in order to detect the presence of specific antibodies on Salmonella Enteritidis in egg yolks are carried out intensively.

The presence of specific antibodies for Salmonella Enteritidis is detected in egg yolk samples from vaccinated flocks resulted in specific positive for a total of 72.22%. Egg yolk samples originating from hens of an unknown immunologic status were cELISA positive in a total of 1.66%. However, egg yolk samples from non-vaccinated hens were positive on the presence of specific antibodies for Salmonella Enteritidis in 23.07% cases.

Bearing in mind that standard bacteriological methods did not confirm the presence of Salmonella Enteritidis in egg yolk samples and that cELISA did establish the presence of specific antibodies in the tested samples it can be concluded that cELISA is a more sensitive test.

Key words: cELISA, egg yolk, Salmonella Enteritidis

INTRODUCTION

Competitive ELISA is proven to be a more sensitive diagnostic method when compared to classical bacteriological methods of isolation of *Salmonella* Enteritidis from egg yolk samples. The cELISA can be used in order to determine the presence of specific *Salmonella* Enteritidis antibodies in egg yolks originating from layer flocks previously vaccinated against the aforementioned agent.

Serologic diagnostics is extensively used in the identification of infections caused by Salmonellae, as well as to follow-up the poultry immune response after

vaccination. Besides, it makes it possible to determine the optimal time for poultry vaccination against salmonellosis.

As mammals transfer specific antibodies to their offspring by means of colostrum, so do birds sensibilized with Salmonella spp. antigens after natural infection or vaccination, as they synthesize specific antibodies which are transmitted transovarially. ELISA analysis of egg yolk samples helps the identification of birds sensibilized to Salmonella antigens.

Previous studies have confirmed that specific antibody titer values in egg yolk samples are in a high correlation with values obtained from respective blood serum samples. The obtained correlation levels were within the range from 0.84 to 0.97 (Nikolas and Andrews, 1991). Piela *et al.* (1984) have found in egg yolks lower values for specific antibody titer values during the first and second week after vaccination or exposure to *Salmonella spp.* These results can be explained by the fact that in the egg yolk IgG are dominant, while IgM are present the first few days after immunization, as well as being the consequence of the primary immune response to salmonella antigens (Piela *et al*., 1984).

Results published by Kramerra and Choa (1970) have demonstrated that different poultry species eggs have a small capacity to deposit specific antibodies. Edgvall, Parmann and Jonsonn (1971) have described the implementation of ELISA tests in order to determine the titer values of higher immunoglobulin classes in poultry serum samples. Results (Piela *et al.*, 1984) have shown clear differences in specific antibody concentrations in different yolk layers originating from vaccinated and non vaccinated flocks. Authors have established that IgG classes are present in egg yolks in the highest concentration, whereas IgM classes are found at highest concentrations during the first two weeks of infection or post vaccination. The same authors have described in their studies two similar methods to extract yolks needed for hemagglutination tests. The first method consists in the dilution of egg yolks with PBS in a ratio of 1:1 followed by centrifugation. The supernatant is than carefully separated and used to carry out the test. The second method comprises the treatment of aqueous yolk extract with chloroform diluted at 1:2 in order to extract lipids present in the egg yolk. The presence of specific antibodies present in the egg yolk was detected by the microalbumin test with the *Salmonella* Enteritidis and *Salmonella* Pullorum antigens 9 days after infection of laying hens (Gast and Beard, 1991). By using monoclonal antibodies (ASCII) to detect *Salmonella* Enteritidis by the ELISA method Brigmon *et al*. (1992) have determined the degree of sensitivity and specificity of the ELISA test with monoclonal *Salmonell*a Enteritidis antibodies.

Specific antibodies are detected at large egg yolk dilutions of the tested samples, which with the use of this method has enabled a fast determination of infected flocks (Ekelijn *et al*., 2006). By ELISA test it is possible to identify the presence of specific *Salmonella* Enteritidis antibodies present in egg yolks even after one year (Sachsenweger *et al*., 1994), as in the samples of blood sera of examined chickens (Findik *et al*., 2010).

MATERIALS AND METHODS

The presence of specific *Salmonella* Enteritidis antibodies was studied in egg yolk samples originating from different hybrids, age groups, categories and immune status (vaccinated and non vaccinated flocks). A total of 730 samples was analyzed, out of which 390 were from non vaccinated laying hens aged 40 weeks, 220 samples were from vaccinated laying hens of the same hybrids and age group and 120 samples were from laying hens of different hybrids, age group and unknown immune status.

In our experiment in order to study the presence of specific *Salmonella* Enteritidis antibodies in egg yolk samples a competitive ELISA Flock Check SE (IDEXX) kit was used.

Preparation of egg yolk samples. Egg yolk samples were twice diluted (ratio 1:1 i.e. 100 μL sample + 100 μL solvent) and homogenized before being placed into the bottom of the wells of ELISA Flock Check microtiter plates.

Results were read with the aid of a spectrophotometer. The absorbance of the developed blue color was determined at 650 nm. Interpretation of the obtained cELISA Flock Check SE kit readings was performed according to the given instructions with a double sample rinse, and with samples which represented a positive and a negative control.

According to the given manufacturer's instructions it was required to test every egg yolk sample, including the positive and negative controls, and thereof to calculate the obtained readings according to specific mathematic formulas. Values were calculated as S/N i.e. by dividing the mean absorbance values of the tested sample (S) by the value of the absorbance of the negative control (N). When the calculated value was larger or equal to 0.75 it was considered as negative, if the calculated value was in the range from 0.60 to 0.74 it was classified as doubtful and the test was repeated. However, if the obtained result was smaller or equal to 0.59 the sample was considered to be positive and the obtained result was confirmed by standard methods.

The samples of the egg shell and egg yolk were examined on the presence of pathogenic bacterias by using the classical bacteriological methods for their isolation and identification.

RESULTS

Egg yolk samples from non vaccinated laying hens. Out of the 390 tested egg yolk samples of non vaccinated laying hens the presence of specific *Salmonella* Enteritidis antibodies was determined by cELISA in a total of 90 samples. A total of 300 tested samples had average absorbance values over 0.75 and were considered to be negative. In this set of tests there were no doubtful samples (Table 1, Figure 1).

Table 1. cELISA tast adsorbance values. Egg yolk samples from non vaccinated layer hens

Number of ELISA tested egg yolk samples	Adsorbance		
	≤ 0.59 (positive)	0.60-0.74 (non specific – repeated)	≥ 0.75 (negative)
390	90	0	300

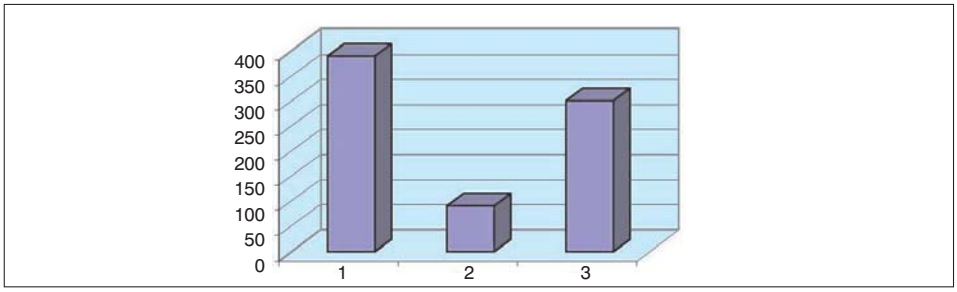

Figure 1. Results of egg yolk samples testing for *Salmonella* Enteritidis specific antibodies in non vaccinated flocks
1. Total number of tested egg yolk samples; 2. Positive samples; 3. Negative samples

Egg yolk samples from vaccinated laying hens. Out of a total of 220 egg yolk samples originating from vaccinated flocks the presence of specific *Salmonella* Enteritidis antibodies was determined in a total of 160 samples whose average absorbance values were 0.59 or less. In 60 samples specific antibodies were not detected as the average absorbance values were 0.75 or over. In this set of trials there were no doubtful readings (Table 2, Figure 2).

Table 2. cELISA tast adsorbance values. Egg yolk samples from vaccinated layer hens

Number of ELISA tested egg yolk samples	Adsorbance		
	≤ 0.59 (positive)	0.60-0.74 (non specific - repeated)	≥ 0.75 (negative)
220	160	0	60

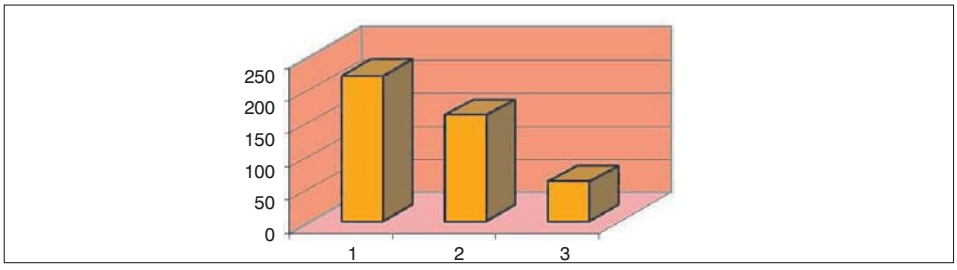

Figure 2. Results of testing for *Salmonella* Enteritidis specific antibodies in egg yolk samples originating from vaccinated flocks
1. Total number of tested egg yolk samples; 2. Positive samples; 3. Negative samples

Egg yolk samples from different hybrids of laying hens, different age groups and unknown immune status. By cELISA testing of a total of 120 egg yolk samples from different hybrids of laying hens, different age groups and unknown immune status the presence of specific *Salmonella* Enteritidis antibodies was determined in only 2 samples. The remaining 118 samples were found to be negative as the absorbance values were 0.74 and higher (Table 3, Figure 3).

Table 3. cELISA tast adsorbance values. Egg yolk samples from layer hens of unknown immunological status, provenience or age

Number of ELISA tested egg yolk samples	Adsorbance	
	≤ 0.59 (positive)	≥ 0.75 (negative)
120	2	118

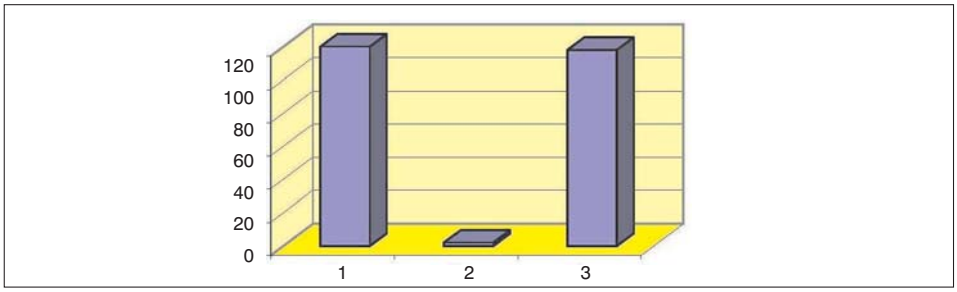

Figure 3. Results of testing for *Salmonella* Enteritidis specific antibodies in egg yolk samples from layer hens of unknown immunological status, provenience or age
1. Total number of tested egg yolk samples; 2. Positive samples; 3. Negative samples

Parallel to the studies on specific *Salmonella* Enteritidis antibodies present in egg yolks of vaccinated and non vaccinated laying hens using ELISA Flock Check SE kit we attempted to isolate *Salmonella* Enteritidis on pre-enrichment media, as well as on selective enrichment media.

At the same time, egg shell specimens were taken for bacteriological analysis.

Salmonella Enteritidis were not isolated in the 390 egg yolk samples originating from non vaccinated flocks. However, cultures made from egg shell were in 40 cases *Pseudomonas* spp. positive. After incubation of the 220 seeded egg yolk samples from the vaccinated flock no *Salmonellae* were isolated, thus the samples were considered to be sterile. Hence, it has been proven that specific *Salmonella* Enteritidis antibodies in the studied samples are the result of vaccination and not the result of infection with the above mentioned bacteria. The 120 egg yolk samples of eggs originating from layer hens of different lines, age groups and unknown immune status no *Salmonellae* were isolated. Cultures made from egg shell were in 20 cases Pseudomonas spp. positive.

Results of studies on the presence of *Salmonella* Enteritidis in egg yolk and shell samples performed by standard bacteriologic methods are shown in Table 4 and Figure 4.

Table 4. Results of the presence of *Salmonella* spp, as well as other bacteria in egg yolk and egg shell samples by using classical microbiological methods

Sample	Vaccinated birds				Non vaccinated birds				Birds of unknown immune status			
	Number of tested samples	*Salmonella* species	Other *Enterobacteriae*	*Pseudomonas* species (egg shell)	Number of tested samples	*Salmonella* species	Other *Enterobacteriae*	*Pseudomonas* species (egg shell)	Number of tested samples	*Salmonella* species	Other *Enterobacteriae*	*Pseudomonas* species (egg shell)
Egg yolks	220	0	0	0	390	0	0	0	120	0	0	20
Egg shells	220	0	0	0	390	0	0	40	120	0	0	0

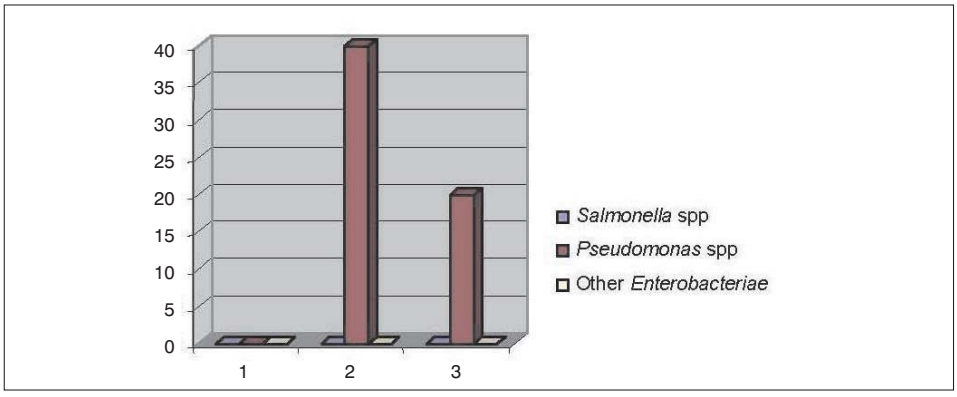

Figure 4. Results of the presence of Salmonella spp, as well as other bacteria in egg shell and yolk samples originating from laying hens
1. Vaccinated flocks; 2. Nonvaccinated flocks; 3. Flocks of unknown immune status

DISCUSSION

In 1969 the term egg yolk immunoglobulin, named as IgY, was introduced for the first time. During the following decade it was established that IgY differs

from class G immunoglobulin present in mammals both in structure and physiological functions. During the 90'ties it was discovered that IgY are more resistant to physical and chemical environmental changes when compared to other classes of immunoglobulins. Until the beginning of the 21 century it was not clear against which antigens IgY are synthesized, bearing in mind that antibodies present in layer hen egg yolks can bind to antigens of a number of bacteria with which the birds come into contact. Since 2002. a number of studies have been carried out in order to determine the role of *Salmonella* spp. in the induction of the synthesis of this class of immunoglobulin by infected birds.

Depending on the methodology, results of the tests on same samples could differ significantly. That depends on the antigen that has been used in order to cover the polystyrene ELISA plates. Most often, ELISA kits are based on bacterial lypopolisaccharide (LPS) antigen that is pre-fixed on to the bottom of the well. Flagellar gm antigen, as well as deflagellar whole bacterial *Salmonella* Enteritidis cells - DEWC antigen (Muzimoto *et al.*, 2006; Ekelijn *et al*., 2006), as antigens are in use. Peilla *et al*. (1984) showed us that in egg yolk originated from laying hens that were infected with Salmonella, besides IgY, one can readily find a significant level of IgG, whereas concentration of IgM can vary, depending on the time period of examination.

ELISA kits that have been studied can be used only as a screening test. However, their advantage is that they can detect birds that have a high level of anti-*Salmonella* Enteritidis antibodies in egg yolk samples and in the blood sera of examined poultry (Van Zijadervald, 1993; Findik, 2010). In order to clarify whether a bird is immune as a consequence of vaccination or infection after use ELISA Flock Check SE kit, one has to perform classical microbiology tests (Brigmon, 1995). In Europe, Denmark was one of the first countries to set a routine testing scheme by cELISA (Feld *et al*., 2000). This competitive immunoenzyme diagnostic kit gives the least inconclusive results. Most often, such immunoenzyme methods use a specific marker protein originating from *Salmonella* Enteritidis, so called Flic-specific 9-kDa polypeptide which acronym is SEP9 (Mizumoto et al., 2006). However, manufacturers of such diagnostic ELISA kits, insists flocks to be vaccinated with the marker protein immunogene i.e. vaccine that is produced by the same pharmaceutical company.

Results of the test using cELISA showed 23.07% positive samples originating from non-vaccinated flocks, whereas 76.93 % of the egg yolk samples were negative. This results are in accordance with others Gast *et al*. (1997) confirming that cELISA has a better sensitivity value for infected birds detection in comparison with standard bacteriological diagnostic methods.

Competitive ELISA showed that specific anti-*Salmonella* Enteritidis antibodies in egg yolks originating from the vaccinated flock were present in 77.22% of samples. Our results are in accordance with other laboratories. Relatively low level of sensitivity of cELISA is the reason why specific anti - *Salmonella* antibodies can not be detected in egg yolks from all birds originating from vaccinated flocks.

Competitive ELISA results of specific anti-*Salmonella* Enteritidis antibodies presence in egg yolk from birds with unknown immune status show that 1.66% of

the samples were positive i.e. have the specific immunoglobulin. Results of our work are in accordance with other authors (McMullin *et al*., 1997).

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ISPITIVANJE PRISUSTVA SPECIFIČNIH ANTITELA NA *SALMONELLA* ENTERITIDIS U ŽUMANCETU JAJETA KOKOŠI NOSILJA KOMPETITIVNOM cELISA METODOM

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SADRŽAJ

Jedan od najčešćih uzročnika salmoneloze ljudi i živine je *Salmonella* Enteritidis. Pomenuti uzročnik se često nalazi u digestivnom sistemu odrasle živine koja ne pokazuje klinički manifestne simptome oboljenja odakle se izlučuje u spoljašnu sredinu fecesom. Ispitivanja se sprovode kako primenom standardnih metoda bakteriološke dijagnostike koje obuhvataju izolaciju *Salmonella* spp. iz žumanceta kokošijeg jajeta i njihovu serološku tipizaciju metodom aglutinacije na pločici. Takođe se pored klasične aglutinacije u mikrotitracionim pločama vrše is-

pitivanja imunoenzimskom probom - ELISA, radi otkrivanja prisustva specifičnih antitela na *Salmonella* Enteritidis u uzorcima žumanceta jaja kokoši nosilja.

Naša ispitivanja su imala za cilj da provere valjanost kompetitivne imunoenzimske probe - cELISA za utvrđivanje prisustva specifičnih antitela protiv navedenog uzročnika u uzorcima žumanaca jaja poreklom od kokoši nosilja.

Prisustvo specifičnih antitela za *Salmonella* Enteritidis ustanovljeno je primenom cELISA kod 72,22% ispitanih uzoraka žumanaca jaja poreklom od vakcinisanih jata i kod 1,66% uzoraka poreklom iz jata nepoznatog imunološkog statusa. Kod 23,07% ispitivanih uzoraka žumanaca jaja poreklom od nevakcinisanih jata ustanovljeno je prisustvo specifičnih antitela za *Salmonella* Enteritidis primenom metode cELISA.

S obzirom da klasičnim bakteriološkim metodama izolacije nije ustanovljeno prisustvo *Salmonella* Enteritidis u ispitivanim uzorcima, a da je primenom metode cELISA utvrđeno prisustvo specifičnih antitela, može se zaključiti da je cELISA osetljivija dijagnostička metoda.