Bangladesh Journal of Veterinary and Animal Sciences, Vol. 5, January 2017

ISSN 2227-6416

Bangladesh Journal of Veterinary and Animal Sciences

Journal home page: www.cvasu.ac.bd/journal/userlist

Research Article

Levels of Ovo-transferrin concentrations in layer chickens experimentally infected with wild-type or small-colony variants of *Streptococcus equi* subsp. *zooepidemicus* via different routes

Roy, K.[1*], Pors, S. E.[2], Christensen, J. P.[2], Biswas, P.K.[1] and A. M.[2] Bojesen[2].

[1]Department of Pathology and Parasitology, [1]Department of Microbiology and Veterinary Public Health, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Chittagong-4225, Bangladesh. [2]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Copenhagen, Denmark.

ARTICLE INFO

Article history :
Submitted date: 20/12/2016
Accepted Date: 26/12/2016

Keywords :
Ovo-transferrin, layer, *Streptococcus zooepidemicus*, intra-venous, intra-tracheal.

** Corresponding Author :*
Phone: +88 01796299974
Email: krisnaroy4@gmail.com

ABSTRACT

Ovo-transferrin is a positive acute-phase-protein in chickens, and a possible biomarker of inflammation and infection. To investigate the level of serum-OvoTransFerrin (OTF) during infectious disease we characterized the OTF levels in brown layer chickens inoculated with a wild-type (WT) or a small-colony-variant (SCV) of *Streptococcus equi* subsp. *zooepidemicus* via the intravenous (IV) or intra-tracheal (IT) routes. Four experimental groups were formed; WT-IV, SCV-IV, WT-IT and SCV-IT each containing 11 birds. From each group, two birds were euthanized on day 1 and day 3 post-inoculation (p.i.), respectively, and remaining birds were euthanized on day 14 p.i. Two hypotheses were tested: 1. OTF levels in inoculated birds were above the day 0 and/or the negative-control bird concentrations, independent on strain and inoculation route. 2. OTF levels varied according to the bacterial phenotype and route of inoculation. The OTF concentration was measured by a commercial Chicken-OTF-ELISA. The median of OTF concentrations (mg/ml) was increased at day 14 p.i. in WT-IV (median = 5.7, range = 4.4 to 7.0), WT-IT (5.3, 3.1 to 7.6) and SCV-IV (6.5, 5.8 to 6.7) groups when compared to corresponding day 0 concentrations (3.0, 2.0 to 3.6; 2.5, 1.8 to 3.6 and 2.1, 0.8 to 2.9 mg/ml, respectively) (P=0.01). In the WT-IV group, the median concentration at day 14 p.i. was higher than the corresponding control (2.9, 2.3 to 11 mg/ml) (P=0.05). In the SCV-IT, there was an insignificant difference between the medians of day 0 and day 14 p.i., however, two birds in the group had the highest OTF concentrations - one at day 1 (9 mg/ml) and another at day 14 p.i. (10 mg/ml). There were no significant differences in the median OTF concentrations when comparing between the experimental groups. The results indicate that the OTF response in layer chickens reflects the inflammatory state. However, the OTF level did not evolve in a strain and/or inoculation route dependent manner.

To cite this paper : *Roy, K., Pors, S. E., Christensen, J. P., Biswas, P.K. and A. M. Bojesen. 2017. Levels of Ovo-transferrin concentrations in layer chickens experimentally infected with wild-type or small-colony variants of Streptococcus equi* subsp. *zooepidemicus via different routes. Bangladesh Journal of Veterinary and Animal Sciences, 5 (1): 17-23*

1. INTRODUCTION

Acute phase proteins (APPs) are a group of blood proteins that are involved in restoring homeostasis by restricting growth of microorganisms, inflammation, surgical trauma or stress through an antibody-independent response, known as an acute phase response (APR) (Murata et al., 2004). An increase of ≥25% of an APP from its basal concentration could be an indication of an undergoing inflammation or a disease process (Gabay and Kushner, 1999; Kushner and Rzewnicki, 1999). A typical major APP following stimulation is detectable for several days and typically

reaches maximal serum concentration within 24 to 48 hours, whereas the resolution happens within four to seven days after the initial stimulus given and if there are no further stimuli (Petersen et al., 2004). The resolution can occur in response to treatment or self-recovery (Xie et al., 2002a). However, the responses can vary depending on the nature of the stimulus e.g. type of invading organism, routes of inoculation and infective dose (Jacobson, 1996).

Ceruloplasmin, C-reactive protein, fibrinogen, fibronectin, haemopexin, haptoglobin, mannan binding proteins, metallothionein, ovo-transferrin, serum amyloid A, transferrin and α_1- acid glycoprotein are considered as acute phase proteins in chickens (Georgieva et al., 2010), yet only limited information has been acquired from experiments in chickens.

Ovo-transferrin (OTF) has been found to be a moderate (2-10-fold increase) (Murata et al., 2004) to major (>10-fold increase) (Xie et al., 2002a) positive acute phase protein (pAPP) in chickens, and indicated as possible biomarker of inflammation and infection in several studies (Xie et al., 2002a, Xie et al., 2002b, Murata et al., 2004, Rath et al., 2009). However, blood profiles of the APP in birds have mostly been studied in response to aseptic inflammation, for example, using croton oil, olive oil, turpentine and lipopolysaccharide (Hallquist et al., 1994, Xie et al., 2002a, Xie et al., 2002b). Xie et al., (2002b) described OTF elevation in chicken serum in response to an *Escherichia coli* infection, yet the dynamics of OTF in chicken serum following a Gram-positive bacterial infection has not been reported.

Streptococcus equi subsp. *zooepidemicus (S. zooepidemicus)* is a pathogenic bacterium in adult chickens, where the clinical signs are associated with respiratory disturbances, fever and diarrhoea lasting for several weeks leading to typical acute or sub-acute septicaemia and can cause up to 80% mortality (Bisgaard et al., 2012). Recently, the pathogenic potentials of a wild-type (WT) and a small-colony variant (SCV) of S. zooepidemicus were shown (Roy et al., 2013), using strains isolated from a natural outbreak in a layer farm (Bisgaard et al., 2012). From our previous study (Roy et al., 2013), it was assumed that the septicaemic condition induced an APR - to restrict or to eliminate the bacterial infection in association with specific host defence mechanisms. Again, different concentrations of OTF were observed in the serum samples used for evaluating the analytical and overlap performances of a commercial Chicken-OTF ELISA kit, where the sera were collected from the chickens infected with the WT or SCV inoculated via the intravenous or intra-tracheal route (Roy et al., 2014).On the basis of assumption drawn from the investigation (Roy et al., 2013) and findings from the study (Roy et al., 2014) it was suggested that the APR or the specific concentration of APPs might vary depending on the *S. zooepidemicus* phenotypes (WT vs. SCV) and their routes of introduction into the chickens.

In the present study we characterized and compared the levels of serum-OTF in brown layer chickens as responses to *S. zooepidemicus* phenotypes -WT and SCV, having inoculated via the intravenous or intra-tracheal routes to test two hypotheses:
Hypothesis 1: All experimental infections induce an APR represented by an OTF elevation above healthy and/or control birds concentrations. Hypothesis 2: The OTF level depends on the strain phenotype and the route of inoculation.

2. MATERIALS AND METHODS

2.1 Sources of samples

To investigate the two hypotheses, serum samples from a previously performed infection study in brown layer chickens were analysed (Roy et al., 2013). All the birds used for the study were free from apparent clinical diseases. All experimental procedures and animal management protocols were undertaken in accordance with legislation of Ministry of Justice, Denmark (Approval number: 2008/561-1481).
Briefly, a *S. zooepidemicus* wildtype (WT) strain (strain F122 Hj5) and a small-colony-variant (SCV) strain (strain F122 Abscess 115) were inoculated either intravenously (i.v. or IV) or via the intra-tracheal (i.t. or IT) route in 45-week old chickens. After one week of acclimatization, each bird was bled through the brachial vein using a 23-G butterfly needle to collect one millilitre (ml) of blood into a vacuum tube (BD Vacutainer™) containing 3.2% tri-sodium citrate for bacteriological examination, and one ml into a plain vacuum tube (BD Vacutainer™) to assess serum-OTF concentration. Then, the chickens were randomly divided into four experimental groups, referred to as WT-IV, WT-IT, SCV-IV and SCV-IT, respectively, depending on the bacterial phenotype and the route of infection. Each group comprised of 11 chickens, whereas two uninfected control groups, "Control-IV" and "Control-IT" contained six and five chickens, respectively. Each group of chickens were housed separately and provided *ad libitum* feed and water. Subsequently, chickens belonging to WT-IV and SCV-IV

groups were inoculated i.v. with 0.50 ml bacterial suspension (10^9 colony forming unit/ml in brain heart infusion broth) of the WT and SCV strain, respectively. Chickens belonging to the WT-IT and SCV-IT groups were injected with a similar dose via the i.t. route. Each of the control birds received 0.50 ml sterile brain heart infusion broth via the i.v. or i.t. routes, respectively. At day one post-inoculation (p.i.) two birds from each group were randomly selected, euthanized and submitted to post-mortem examinations. Two additional birds were examined at day 3 p.i., whereas the remaining birds were examined at day 14 p.i. Prior to euthanization, one ml blood was collected from each bird for assessment of OTF concentration, and further one ml was collected from the i.v. inoculated birds for bacteriological examination. A tracheal swab was obtained from the i.t. inoculated birds at day 14 p.i. to enable bacterial re-isolation of the inoculated strains. From all birds, organ swabs were obtained from livers, spleens and other organs upon indication.

A complete dataset could not be obtained from two birds in the WT-IV group, and four birds from the SCV-IV group, due to sudden death. The day zero (day 0) samples were considered to represent the basal level of OTF in healthy birds of each group prior to the inoculations. The maximal OTF concentration at day 0 in each group including the control was considered as the baseline to identify the chickens with altered serum-OTF levels in response to the inoculation of the two different phenotypes via either the i.v. or i.t. routes.

2.2 Serum preparation and quantification of serum-OTF level

For separation of serum from a blood sample centrifugation was done at 25°C for 30 minutes at 2415g. To quantify serum OTF level (mg/ml) in the chickens, we used the commercially available Chicken-OTF-ELISA (ICL, Inc.®, E-30TX)- an Enzyme-linked immunosorbent assay, previously evaluated, where we followed an adjusted two-step dilution method with a final dilution of 1:63,001 (serial-251 fold dilutions)(Roy et al., 2014). The levels of serum-OTF were assessed on the basis of its concentration (mg/ml) in the serum samples collected at different days' post-inoculation.

2.3 Clinical signs, gross lesions and bacteriology

Clinical signs were recorded during the trial and immediately prior to euthanization of the birds. Gross lesions were recorded while performing necropsy. The procedures employed for bacteriological culture and identification have recently been described (Roy et al., 2013). Clinical signs, gross lesions and bacteriological re-isolation rates with the levels of serum-OTF concentrations observed at specified days' post inoculations were indicative parameters to correlate the intensities of undergoing inflammation process attributable to the phenotypic variants inoculated by two different routes. To link clinical signs, gross lesions and bacteriological re-isolation rates to the OTF levels in the individual birds we compared the OTF concentrations from the present study with the previously our published results concerning clinical signs, gross lesions and bacteriological re-isolation rates (Roy et al., 2013).

2.4 Justification of hypotheses and statistical analysis

To test hypothesis-1, the serum-OTF concentrations obtained at the specified days in each experimental group were compared with the day 0 concentrations for each bird and the corresponding serum-OTF concentrations in the control birds. To test hypothesis-2, the OTF concentrations were compared between the experimental groups at each of the consecutive sampling days for each group to enable assessment of differences in the group-characteristic OTF levels. The Mann-Whitney test was used to compare the differences between the medians of concentrations at day 0 and day 14 p.i. within each group (hypothesis-1) and at day 14 p.i. between the groups (hypothesis-2). All tests were done at a 95% confidence level. The GraphPad Prism software (Version 5, GraphPad Software, San Diego, California, USA) was used for data analyses.

3. RESULTS

Elevated serum-OTF concentrations of different magnitudes were observed in all but SCV-IT experimental group, whereas basically no changes were observed in the control chickens. Birds of the control group did not show any clinical signs and gross lesions and remained culture-negative to *S. zooepidemicus* during the entire experiment.

3.1 Serum-OTF concentrations

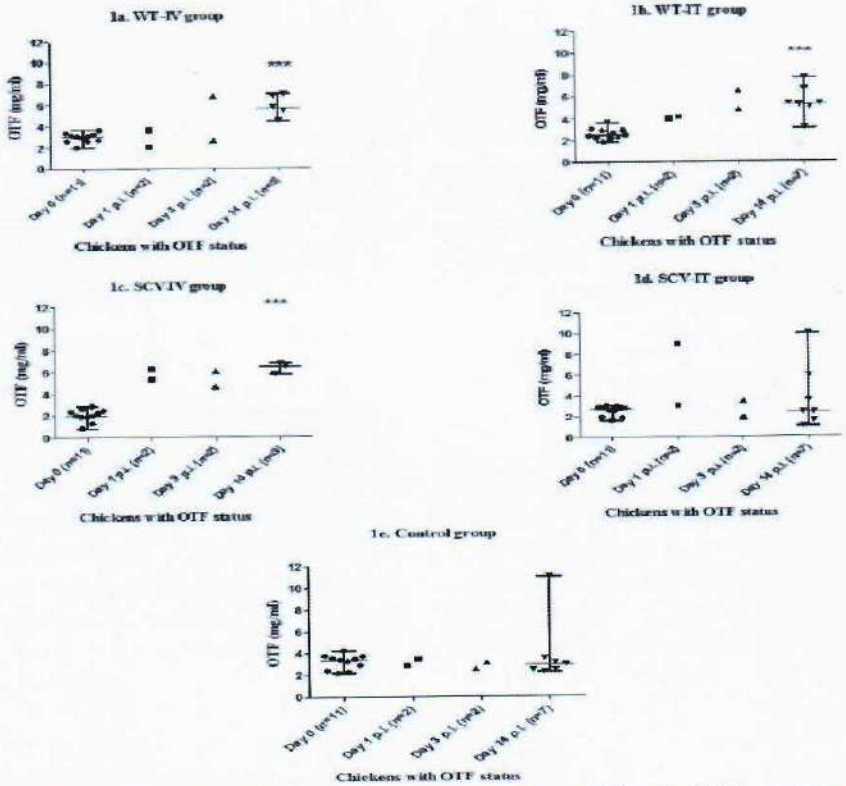
The OTF levels has been shown in Figure 1 (a to e). In the WT-IV group (1a), the median of OTF concentration was approximately two times higher at day 14 p.i. compared to day 0 (P=0.01). One chicken out of two at each of day 1 and day 3 p.i. and all birds of day 14 p.i. had an elevated OTF concentration when compared to the baseline concentration. In the WT-IT group (1b), the median OTF concentration was two times higher at day 14 p.i. compared to the day 0 (P=0.01). All birds,

except one at day 14 p.i., were recorded with elevated OTF concentrations. In the SCV-IV group (1c), the median OTF concentration was three times higher at day 14 p.i. compared to the day 0 (P=0.01), and all the experimentally infected birds had elevated OTF concentrations. In the SCV-IT group (1d), there was no significant difference between the median concentrations of day 0 and day 14 p.i. (P=1.0). There was no significant difference between the medians OTF concentrations of the different groups when compared on a day 14-by-day 14 basis. In the control (1e), the OTF concentrations remained at a low level during the entire trial with an insignificant difference between the day 0 and day 14 p.i. medians (P=0.65). All individual OTF concentrations were under the baseline value in the control birds except for one bird at day 14 p.i. There was a significant difference between the median concentrations of the control and the WT-IV group at day 14 p.i. (P=0.05).

3.2 Clinical signs, gross lesions and bacteriological re-isolation rates compared to the serum-OTF levels

The clinical signs, gross lesions and bacteriological re-isolation rates have previously been reported by Roy *et. al.*, (2013). Briefly, all birds of the WT-IV group showed signs of depression during the entire experimental trial. Lowered body condition (5/5) and a soiled cloacal region (4/5) were also observed at day 14 p.i. The birds of WT-IT also had a lowered body condition (5/7) and a soiled cloacal region (3/7) at day 14 p.i. Only two birds in the SCV-IT group had a soiled cloacal region at day 14 p.i, and no clinical signs were observed in the birds from the SCV-IV group.

The gross lesions observed in the birds are summarized in Table 1. Briefly, in the WT-IV group, ovary, spleen and kidney were recorded with gross lesions in the bird at day 1 p.i., which also had an elevated OTF concentration. A similar pattern was recorded in birds at day 3 and day 14 p.i. In the WT-IT group, the birds with elevated OTF concentrations also had gross lesions in different organs, except for one bird at day 14 p.i. In the SCV-IV group, all birds at day 14 p.i. having elevated OTF concentrations were recorded with gross lesions in multiple organs. In the SCV-IT group, the birds at day 1 p.i. having elevated OTF concentration also had gross lesions in the lungs (2/2) and kidney (1/2). In the same group, only lung lesions were recorded in the bird examined at day 3 p.i. and lesions in different organs were found in two birds at day 14 p.i. having elevated OTF concentrations.



*(***, significant difference with the median concentrations of day 0; Different sizes dots, samples or observations; Mid horizontal lines, median of OTF concentrations; p.i., post inoculation; Two small lines with the median line, Range, that is minimum and maximum concentrations)*

Fig. 1: Illustrative scattered dot plots are showing the ovo-transferrin (OTF) concentrations(mg/ml) in layer chickens of five groups: WT-IV (1a), WT-IT (1b), SCV-IV (1c), SCV-IT (1d) and negative control (1e), where the first two groups were infected with the wild-type (WT) of *Streptococcus equi* subsp. *zooepidemicus* via the intravenous (IV) and intra-tracheal (IT) routes, respectively, while the third and fourth groups, respectively, via the IV and IT route with the small-colony variant (SCV) of the organism. The dynamics of OTF in the infected chickens is shown displaying the serum-OTF levels as observed at day 1, 3 and 14 post infections, and its level in the chickens prior to the inoculation (day 0) is in Figure 1 (a to d), and in the control group in Figure 1e. The baseline concentrations were 3.6, 3.6, 2.9, 3.0 and 4.3 mg/ml in the WT-IV, WT-IT, SCV-IV, SCV-IT and control groups, respectively, considered from the maximum concentrations of corresponded day 0 samples.

Table 1. The organs showing gross lesions in layer chickens inoculated with wild-type or small-colony variant of *Streptococcus equi* subsp. *zooepidemicus* via intravenous or intra-tracheal route.

	Wild-type						Small-colony variant					
	Intravenous			Intra-tracheal			Intravenous			Intra-tracheal		
	Day 1[a]	Day 3	Day 14	Day 1	Day 3	Day 14	Day 1	Day 3	Day 14	Day 1	Day 3	Day 14
Euthanized birds	2	2	5	2	2	7	2	2	3	2	2	7
Organs positive to gross lesions	2/2[b] KD: 2 OV: 1 SP: 1	2/2 KD: 2 SP: 2 AC: 2 OV: 1 LV: 1	5/5 SP: 5 OV: 5 LV: 3 KD: 1	2/2 LU KD SP	2/2 LU: 2 KD: 2 OV: 2 SP: 2 LV: 1	6/7 SP: 6 OV: 6 LV: 4 AC: 3 KD: 1	0	0	3/3 SP: 3 OV: 3 LV: 1 KD: 1	2/2 LU: 2 KD: 1	2/2 LU: 2 OV: 1 LV: 1	2/7 LU OV SP AC

AC, cervical air-sac with inflammation and with/without fibrino-purulent appearance; KD, kidney with nephropathy; LU, lung with broncho-pneumonic lesions; LV, liver with enlargement and/or necrosis and/or peri-hepatitis; OV, ovary with regression and/or oophoritis associated with peritonitis and with/without chronic adhesive fibrino-purulent appearance; SP, spleen with enlargement and/or necrosis and/or proliferation of white pulp; ND, not done; [a] Day 1, day one post-infection, and similarly Day 3 and Day 14; [b] Frequency of chickens showing gross lesions in an organ/total number of chickens.

All the WT infected chickens were found culture-positive to the inoculated strain in one or more organ systems during the entire trial; in the WT-IV group this was spleen (2/2) and liver (1/2) at day 1, spleen (2/2) and cervical air-sac (2/2) at day 3, and spleen (5/5) and liver (4/5) at day 14 p.i.,in the WT-IT group this was spleen (2/2), lung (2/2) and liver (1/2) at day 1, spleen (2/2) at day 3, and spleen (5/7), liver (7/7) and peritoneum (2/7) at day 14 p.i. All the birds in the WT-IV group were also positive in the blood cultures except for two at day 14 p.i. Some birds (4/7) in the WT-IT group were culture-positive from tracheal swabs. The chickens of the SCV-IV group were culture-positive from spleen samples (2/2) at day 3 p.i., and spleen (1/3) and liver samples (1/3) (same bird) at day 14 p.i. In the SCV-IT group a positive culture from the spleen (1/2) and lung (1/2) from one bird at day 3 p.i, and cervical air-sac (2/7) and lung (1/7) at day 14 p.i. were culture-positive to the SCV strain. No birds were positive in the swab cultures at day 1 p.i. in either of the SCV groups. Only birds sampled at day 3 p.i. were positive in the blood cultures following SCV-IV (2/2) inoculation. Most birds (5/7) were positive from the tracheal swabs in the SCV-IT group at day 14 p.i.

The two birds having elevated OTF concentrations at day 1 p.i. in the SCV-IV group did not show clinical signs or gross lesions and remained culture-negative. Additionally, the birds in the same group sampled at day 3 p.i. did not show clinical signs or gross lesions but remained culture positive from blood and organ swabs. Again, one SCV-IT bird assessed at day 14 p.i., which had an elevated OTF concentration, was also recorded with no clinical signs or gross lesions yet remained culture positive from the tracheal swab. All the birds tested at day 0 were culture-negative.

4. DISCUSSION

We found an elevated OTF concentration in three out of the four experimental groups. Hence, hypothesis 1 was proven by showing a significant elevation of median OTF concentration at day 14 p.i. compared to the corresponding day 0 concentrations in the WT-IV, WT-IT and SCV-IV groups. Few infected birds in the SCV-IT group were recorded with increased OTF concentrations, whereas all infected birds in the SCV-IV group had elevated concentrations. The variation between groups may be due to the less chance of SCV to disseminate internally in the birds inoculated via the i.t. than i.v. route and thereby inducing an APR (Roy et al., 2013). The results did not support hypothesis 2, since there were insignificant differences when we compared the median OTF concentrations between the experimental groups. This finding indicates that the APR did not vary according to the type of inoculated strains or their routes of inoculation. The insignificant differences between median concentrations and a generally low OTF concentration in the control groups indicated that blood sampling and handling of the bird alone did not induce a significant OTF response.

Although, there were insignificant differences between experimental groups in the median OTF concentrations, the increased serum-OTF level from ~ 3 mg/ml at day 1 to ~ 6 mg/ml at day 14 p.i. in the group WT-IV, as opposed to the declining OTF levels in the SCV-IT group may reflect differences in the host clearing patterns. Hence, in case of the WT-IV group, the increasing OTF level could be due to a progression of the infection in a host unable to handle it, whereas the opposite situation may have applied in the chickens of the SCV-IT group. This somewhat agrees with the findings by Petersen et al., (2004), indicating that accelerated clearing over time impacts the OTF concentration and allows the use of OTF as non-specific biomarker. Individual bird's physiology, however, could also play a role. The highest OTF concentrations were observed at day 14 p.i. in each experimental group thus indicating an on-going infection, which was supported by our clinic-pathological findings previously reported (Roy et al., 2013). Consequently, the results of the present study also showed the reason to consider using up to day 14 p.i. for detecting the level of OTF.

A very few birds' serum samples were investigated at day 1 and day 3 p.i. compared with that of day 14, the end of the experiment to show the difference in OTF level between very early- and late stage of APR. The small sample sizes for those two specified days p.i, were although a limitation of this study, the major aim was however to assess the level of OTF at the late stage of an undergoing infection induced by two different phenotypes of *S. zooepidemicus*, to have an idea whether its level can be a biomarker in view of diagnosing late or chronic infection caused by the organism. To collect blood sera thus we euthanized a higher number of birds at day 14 p.i compared with day 1 and 3. The results of significant increase of OTF at day 14 p.i in the three experimental groups except SCV-IT is, however, not conclusive that the highest peak of OTF can be seen at day 14 p.i until a further study of a longer duration is conducted.

Gross lesions in ovaries, spleens, livers, lungs, cervical air-sacs and kidneys were generally found to correlate well with elevated serum-OTF levels and only one bird from each of the WT-IT and SCV-IT groups was recorded with a slightly elevated OTF concentration in the absence of gross lesions but having positive cultures from the tracheal swabs. In addition, two birds in the SCV-IV group at day 1 p.i. did not show any clinical sings and gross lesions, and were also negative to the bacterial cultures; however, the OTF concentration was considerably elevated compared to the day 0 and the

controls. This may be due to the SCVs entering a dormant state or that the bacterial cells may hide intracellularly (Sendi and Proctor, 2009, Roy et al., 2013). In the SCV-IT group, most of the chickens had a serum OTF concentration below or equal to the baseline. Interestingly, two of the birds in the SCV-IT group had the highest OTF concentrations in the study and both birds were suffering from bronchopneumonia. Intracellular adaptation of SCVs, macrophage insufficiency orability to cause recurrent infections might be the reasons for this finding (Sendi and Proctor, 2009, Roy et al., 2013).

5. CONCLUSION

In conclusion, the serum-OTF concentration was elevated in three out of four groups of *S. zooepidemicus*-infected layer chickens. No differences were recorded between strains and routes of inoculation indicating that the serum-OTF concentration in the *S. zooepidemicus*-infected layer chickens is not affected significantly by the streptococcal phenotype or the route of inoculation. However, a bigger sample size and a higher number of sample points are warranted to confirm the individual results and to fully characterize the kinetics of serum-OTF in layer chickens.

ACKNOWLEDGEMENTS

The authors would like to thank University of Copenhagen for funding. Thanks to Professor Mads Kjelgaard-Hansen and laboratory technicians Claus Stjernegaard and Lisa Lovenbo (Central Laboratory of Veterinary Clinical Pathology) from Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, for excellent technical assistance in laboratory.

REFERENCES

Bisgaard, M., Bojesen, A.M., Petersen, M.R. and Christensen, H. 2012. A major outbreak of *Streptococcus equi* subsp. *zooepidemicus* infections in free range chickens is linked to horses. Avian Diseases, 56: 561-566.

Gabay, C. and Kushner, I. 1999. Acute phase proteins and other systemic response to inflammation. The New England Journal of Medicine, 340: 448-454.

Georgieva, T.M., Koinarski, V.N., Urumova, V.S., Marutsov, P.D., Christov, T.T., Nikolov, J., Chaprazov, T., Walshe, K., Karov, R.S., Georgiev, I.P. and Koinarski, Z.V. 2010. Effects of *Escherichia coli* infection and *Eimeria tenella* invasion on blood concentrations of some positive acute phase proteins (haptoglobin (PIT 54), fibrinogen and ceruloplasmin) in chickens. Revue de Medecine Veterinaire, 161: 84-89.

Hallquist, N.A. and Klasing, K.C. 1994. Serotransferrin, ovotransferrin and metallothionein levels during an immune response in chickens. Comparative Biochemistry and Physiology, 108B: 375-384.

Jacobson, R.H. 1996. Principles of validation of diagnostic assays for infectious diseases. Manual of Standards for Diagnostic Tests and Vaccines, 8-15 (OIE).

Kushner, I. and Rzewnicki, D. 1999. Acute phase response, in: Gallin, J.I. and Snyderman, R. (Eds.) Inflammation: basic principles and clinical correlates, pp. 317-329 (Philadelphia, Lippincott, Williams and Wilkins).

Murata, H., Shimada, N. and Yoshioka, M. 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. The Veterinary Journal, 168: 28-40.

Petersen, H.J.P., Nielsen, P. and Heegaard, M.H. 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Veterinary research, 35: 163-187.

Rath, N.C., Anthony, N.B, Kannan, L., Huff, W.F., Huff, G.R., Chhapman, H.D., Erf, G.F. and Wakenell, P. 2009. Immunology, health, and Diseases: Serum ovotransferrin as a biomarker of inflammatory diseases in chickens. Poultry Science, 88: 2069-2074.

Roy, K., Bisgaard, M., Kyvsgaard, N.C., Christensen, J.P., Nielsen, O.L., Biswas, P.K., Pors, S.E. and Bojesen, A.M. 2013. Pathogenicity of wild-type and small-colony variants of *Streptococcus equi* subsp. *zooepidemicus* in layer chickens. Avian Pathology, 42: 316-322.

Roy, K., Kjelgaard-Hansen, M., Pors, S.E., Christensen, J.P., Biswas, P.K. and Bojesen, A.M. 2014. Performance of a commercial Chicken-Ovo-transferrin-ELISA on the serum of brown layer chickens infected with *Gallibacterium anatis* and *Streptococcus zooepidemicus*. Avian Pathology, 43: 57-61.

Sendi, P. and Proctor, R.A. 2009. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends in Microbiology, 17: 54-58.

Xie, H., Huff, G.R., Huff, W.E., Balog, J.M., Holt, P. and Rath, N.C. 2002a. Identification of Ovotransferrin as an Acute Phase Protein in Chickens. Poultry Science, 81: 112-120.

Xie, H., Newberry, L., Clark, F.D., Huff, W.E., Huff, G.R., Balog, J.M. and Rath, N.C. 2002b. Changes in Serum Ovotransferrin Levels in Chickens with Experimentally Induced Inflammation and Diseases. Avian Diseases, 46: 122-131.