

Effect of *Staphylococcus aureus* infection on the heat stress protein 70 (HSP70) level in chicken embryo tissues

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ABSTRACT *Staphylococcus aureus* bacteria are components of physiological biocenosis of skin or mucous membranes in some animals' genera but also they are dangerous opportunistic pathogens responsible for infections of various localization, course or manifestations. Proteins produced by these bacteria destroy tissues, leukocytes and cause haemolysis of erythrocytes. Host organisms respond by defence mechanisms. Production of heat stress proteins (HSPs) is one of defence responses of infected host organism. To evaluate infection and host defence mechanisms some animal models of experimental infection are reported. Use of chick embryo model allows demonstrating adequate differences in staphylococcal virulence depending on the strain genotype. The aim of the study was to examine the changes in heat shock protein HSP70 levels in chick embryo tissues after infection caused by *S. aureus* strains no. tu2, pa3, ch5, ch10, ch24, and ch25 isolated from chickens. The

bacteria were injected directly into fluid of amnion cavity and incubated for 10 days. The mortality of particular chick embryos was reported and the tissues for further analysis were taken every day from day 13 to day 19. The levels of heat stress protein HSP70 were determined by dot-blot method. Results showed that the strains no. ch5, ch24, and ch25 were the most virulent. HSP70 levels increased in all groups of injected embryos at the same time the hatching process was started. The presented study showed that the infected chick embryos were characterized by higher HSP level from 12.3% up to 19.7% compared to the control group. The morphological analysis showed numerous erythrocytes with damaged cell membranes and morphological changes of erythrocytes. Changes in the level of HSP70 protein can be a useful indicator of infection caused by *S. aureus* bacteria. Additionally, chicken embryo is a helpful research model in studies of pathogenesis of diseases caused by bacteria.

Key words: in ovo model, staphylococci, defence mechanisms, hatchability

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INTRODUCTION

The phenomenon of the transmission of *Staphylococcus* bacteria between animals and humans is found more and more often (Fluit, 2012; Heaton et al., 2020). For this reason, the search for an appropriate research model for experiments on the etiopathogenesis of infections with these bacteria is being carried out on a wider scale

(Polakowska et al., 2012; Bonar et al., 2016). The use of mammalian models (such as a rabbit or a domestic pig) is expensive and ethically ambiguous (Sanden et al., 1989; Andersen and Winter, 2019). Simultaneously, alternative models such as tissue cultures, cell lines or invertebrate animals were proposed. However, they did not meet the expectations placed in them due to significant discrepancies and the lack of compliance with the results obtained in experiments with vertebrate animals (Polakowska et al., 2012; Międzobrodzki and Kosecka-Strojek, 2018). In this context, it seems that the advantages of mammalian and alternative models are in the chick embryo. It is not only susceptible to infection with *Staphylococcus* bacteria, but it is also characterized by relatively low breeding costs and availability compared

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to mammalian or alternative models (Polakowska et al., 2012; Bonar et al., 2016). Additionally, a chicken embryo develops outside the mother's organism, which facilitates observation and conducting experiments (Scanes and McNabb, 2003).

The course of systemic infection with *Staphylococcus aureus* is associated with the bacterial toxin α (α -haemolysin). It can induce rapid aggregation of platelets in the body leading to the formation of microclots which are mainly retained in the liver. In such a situation, this organ acts as a physical barrier responsible for the filtration of dangerous clots and harmful antigens reaching the body. Hepatic macrophages catch *S. aureus* and then platelets bind to them, preventing the pathogen from spreading further. This can disturb blood circulation within the liver and lead to blood clots. In addition, most acute phase proteins, complement components and cytokines involved in the response to bacterial infection are synthesized in this organ (Surewaard et al., 2018). Infection caused by *S. aureus* causes severe oxidative stress to the host cells, which leads to the formation of free radicals both in the entire body and in the liver (Ramaglia et al., 2004). As a consequence, this results in further disturbances such as: damage to the mitochondria and inhibition of ATP production, disturbance of DNA repair processes or destabilization of lysosomal membranes (Nair et al., 2013).

An organism's exposure to environmental stressors starts a series of biochemical processes aimed at maintaining the homeostasis of the organism. Increased synthesis of Heat Shock Proteins (HSPs) under stress conditions in both *Prokaryota* and *Eucaryota* is a mechanism of constant cellular response and is widely regarded as the leading endogenous manifestation of cell adaptation to environmental changes. They are synthesized in response to various stimuli such as: heat stress, hypoxia, ischemia, viral or bacterial infections, as well as the action of various toxic substances, including heavy metals (Rabczyński et al., 2006; Kantidze et al., 2015).

HSP proteins belong to a family of polypeptides with a high degree of conserved primary structure and can be divided based on their molecular weight. One of the most precise classifications distinguishes: HSP 100 (> 100 kDa), HSP90 (~ 81–99 kDa), HSP70 (~ 65–80kDa), HSP60 (~55–64 kDa), HSP 40 (~ 35–54 kDa), sHSP (< 35 kDa), HSP10 (~10kDa) and Ubiquitin (~8kDa) (Macario et al., 1999). The main role of HSP is to protect intracellular proteins against proteolysis, as well as participate in the post-translational modification, stabilization and secretion of proteins by reorganizing the spatial structure of proteins with incorrect conformation in order to restore their native form. They also support the presentation of antigens and the biosynthesis of antibodies (Każmierczuk and Kiliańska, 2009; Wyżewski et al., 2014).

Regarding the functions in the body, the HSP70, present in cytosol and cell nuclei, is the most important one. The HSP70 is responsible for the dissolution of protein complexes and control of regulatory proteins and cell signalling, as well as translocation of precursor proteins in mitochondria and protection of mitotic cells against

abnormal divisions caused by damage to the centrosome (Li, 2017). HSP70 is encoded by HSPA1A, HSPA1B, and HSPA1L, and unlike other proteins of this subfamily, it undergoes a low constitutive synthesis, while it shows increased expression during stress, especially under conditions of hypothermia, oxidative stress and pH changes (Li et al., 2017). The increase in HSP70 expression is also associated with the anti-inflammatory effect, as this protein reduces the concentration of pro-inflammatory cytokines (IL-6, IL-8), while stimulating the secretion of anti-inflammatory IL-10 (Luo et al., 2008).

The aim of the study was to investigate the defensive reaction of a chicken embryo to *S. aureus* infection by analysing the survival rate of chicken embryos and changes in the haematological picture of blood and the level of heat stress protein HSP70 in the liver during subsequent stages of embryogenesis.

MATERIALS AND METHODS

Experimental Design

According to Directive 2010/63/EU, the experimental and animal procedures used in this study did not need to be approved by the Local Animal Ethics Committee.

Hatching Eggs and Their Incubation

Hatching eggs (egg weight (mean \pm SD) 60.3 \pm 6.11 g) of Ross 308 broiler chicken parental flock (Aviagen) were obtained from a commercial farm (Sławomir Domagała, Gołaczewy, Poland). The eggs were incubated in a Brinsea type OVA-Easy advance incubator under standard conditions, i.e.: temperature 37.8 \pm 0.1°C, relative humidity (RH) 50% \pm 2%. On day of incubation (E) 10th (E10) the eggs were candled with using an ooscope lamp to determine embryo development, and infertile and dead embryos eggs were rejected. The remaining eggs with live embryos (n = 360, 60 eggs per group) were used in further procedures (Table 1).

Microbiological Material

Staphylococcal strains used in the experiment were stored at -80°C on TSB medium containing 10% glycerol, and after revitalization on solid TSA. Bacterial strains were grown overnight in TSB liquid medium at 37°C with shaking (Polakowska et al., 2012).

Approximately 2 hours before the planned injection, the optical density at a wavelength of 600 nm (OD600) of the liquid cultures was measured and equated to the value 8.00 (DTP 8.0). In three consecutive series of centrifugations, bacterial cells were rinsed with the remainder of the culture medium and suspended in sterile 0.7% NaCl solution. At this stage of the study, the cells were stored on ice at 4°C. Then it was checked that all bacterial cell suspensions contained the same number of bacterial cells by plating 50 μ l in duplicate on TSA solid medium and incubating overnight at 37°C. Colonies

Table 1. Schema of groups and description of *Staphylococcus aureus* strains used in experiment.

Strain	Group size [egg]	Bacterial titre in 50 μ L medium	Expected virulence	Origin	References
Control - sterile 0.7% NaCl water solution	60 eggs	0.0	-	-	-
pa3 (B305236/1)	60 eggs	DTP 8.0	weak	(Scotland, UK, 2006) isolated from farmed partridges	van Leeuwen et al., 1996
tu2	60 eggs	DTP 8.0	weak	(Wroclaw, Poland, 2008) isolated from deep wounds in turkeys	Polakowska et al., 2012
ch10 (M1-hen)	60 eggs	DTP 8.0	weak	Isolated from the Bursa of Fabricius of broiler chickens (Iowa, USA, 1999)	van Leeuwen et al., 1996
ch24 (M1-hen)	60 eggs	DTP 8.0	weak	(Wroclaw, Poland, 2008) isolated from deep wounds in broiler chickens	Polakowska et al., 2012
ch5 (CIX38)	60 eggs	DTP 8.0	strong	(Belgium, 1976) isolated from broiler chickens	van Leeuwen et al., 1996
ch25 (strain M2-hen)	50 eggs	DTP 8.0	strong	(Wroclaw, Poland, 2008) isolated from the liver of broiler chickens	Polakowska et al., 2012

grown on the solid media for each dilution were counted in order to determine the value of the colony forming unit (CFU) corresponding to the number of dividing bacterial cells contained in the volume of 50 μ l of each used suspension.

Experimental Setup and Suspension Injection

The first part of the study covered six *S. aureus* strains, which in previous studies (Polakowska et al., 2012) showed low (strains: tu2, pa3, ch10, ch24) and high virulence (strains: ch5 and ch25) for chicken embryos (Table 1).

Injection Procedure

The injection procedure was made at E10 according to the recommendation from Soltesz and Mardh (1986) that chicken embryos show maximum susceptibility to *S. aureus* infection at this stage of embryogenesis.

The embryonated eggs were selected and divided into seven parallel groups (n = 60 eggs per group); the surface of every egg was disinfected with 70% ethanol. Next, a small hole in the shell (diameter of about 2 mm) was made using an 18G needle (NIPRONIPRO MEDICALPOLAND, Warszawa, Poland) at the egg blunt end. The previously prepared bacterial suspensions in 50 μ L of 0.7% saline solution were injected into the amniotic sac according to the previously described method (Polakowska et al., 2012; Bonar et al., 2016). The control group eggs were injected only with sterilized 0.7% saline solution. The injection was made using a 25G needle (NIPRONIPRO MEDICAL POLAND, Warszawa, Poland) and 1 mL syringe (ZARYS International Group Ltd, Zabrze, Poland). After manipulation, the holes were sealed with hot paraffin, and incubation was continued. The eggs were candled daily for seven days to evaluate the viability of embryos.

Monitoring of Embryo Development

Dead embryos were distinguished by the absence of spontaneous movement (usually associated with

haemorrhage), loss of vascular architecture, and abnormal morphology. Such eggs were removed from the incubator and the stage of development was confirmed by break out and analysed using Hamburger and Hamilton's key (1951). Incubation was terminated on the 19th day of embryogenesis (E19) and all live embryos were euthanized.

Tissue and Blood Collection

Every day, starting on 13th day of embryogenesis (E13), tissues (blood and liver) were collected from four embryos from each group. The collected liver samples were placed in Eppendorf tubes and stored at -80°C until further analysis. The blood was collected from the umbilical artery of the embryo based on the method described by Sechman et al. (2006). The egg shell was punctured from the air chamber side, and then the egg was gently opened in a shallow vessel with water at a temperature of about 40°C in order to isolate the embryo together with the foetal membranes, taking care not to damage the blood vessels to isolate the umbilical artery. The dissected artery was clamped with surgical tweezers and then cut. After releasing the clamp, the first clean drop of blood was placed on a glass slide and with one firm movement a smear was made with the bevelled edge glass slide at an angle of approximately 45° so that the blood was spread along its entire surface. Smears were made and stained using the hemactolor staining kit (Cat. No.111661, MERCK Polska Ltd, Warszawa, Poland). After drying, the smears were analysed using a Nikon microscope integrated with the photo camera at 60 \times magnification.

Determination of HSP Proteins in Embryo Tissues by Dot-blot Method

To examine expression of stress proteins, dot-blot assays were performed in a 96-well plate format using a Bio-dot microfiltration manifold (Bio-Rad, Hercules, CA). Homogenates of liver tissues were prepared

Table 2. The mortality of embryos in ovo injected by the suspension with selected *Staphylococcus aureus* strains on the 10th day of incubation (E10).

Group	Control (0.7% NaCl)	pa3strain	tu2 strain	ch10strain	ch24 strain	ch5strain	ch25 strain
Treated eggs [number]	60	60	60	60	60	60	60
Embryos died between E10-E13	14 ^a (23.3%)	16 ^a (26.7%)	12 ^a (20.0%)	15 ^a (25.0%)	30 ^b (50.0%)	32 ^b (53.3%)	30 ^b (50.0%)
Embryos died between E14-E19	2 (3.3%)	0 (0.0%)	5 (8.3%)	0 (0.0%)	2 (3.3%)	2 (3.3%)	6 (10.0%)
Embryos died -total	16 ^a (26.7%)	16 ^a (26.7%)	17 ^a (28.3%)	15 ^a (25.0%)	32 ^b (53.3%)	34 ^b (56.7%)	36 ^b (60.0%)
Sampled embryos [number]	24	24	24	24	24	24	24

The results are presented as number and per cent of died embryos from treated eggs in the following days of incubation: 10-13 (E10-E13) and 14-19 (E14-E19).

^{a,b}Values in the rows marked with different letters differ statistically significantly ($P < 0.05$).

according to the manufacturer's protocols (Roche Applied Diagnostic GmbH, Mannheim, Germany). First, protease inhibitor cocktail PMSF (Roche) was used to prepare tissue extracts. The amount of protein was determined by the BCA method (Sigma-Aldrich), and samples were adjusted to an equal concentration. Subsequently, 50 μ l of samples (35 μ g of protein) was added to each well of the microfiltration apparatus and the blotting was performed according to a standard (Bio-Rad). Then, the membranes were blocked for 60 min at 37°C in a blocking buffer containing 5% non-fat milk solution (Gostyń, Poland) dissolved in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). Next, the membranes were treated with primary monoclonal murine anti-Heat Shock Protein 70 Antibody (3A3) (Cat.No. MA 3-006, ThermoFisher Scientific) solution at a dilution of 1:750 (TBS with 0.05% Tween 20) containing 1% non-fat milk, and incubated overnight at 4°C. The second day of analyses, membranes were washed three times with TBST, and incubated again with the secondary rabbit anti-mouse antibody (Cat. No. 402335, Calbiochem) in dilution 1:1000 for 90 at room temperature. The HSP70 reaction was developed using DAB (3,3'-diaminobenzidine) HRP substrate (Sigma-Aldrich) with addition of imidazole and 3% H₂O₂, followed by a colour reaction. After the reaction was stopped, the membranes were air-dried and a densitometric analysis of protein dots was performed using a Syngene Ingenius instrument using the GeneSnap software.

Due to the risk of cross-reaction or high background, in order to match the antibody dilutions, test determinations were made on 2 controls and 4 experimental ones.

Statistical Analyses

The hatchability results were analysed by a z-test, while the results of the level of the HSP70 were subjected to a two-way ANOVA followed by Tukey's multiple range test. The relation between level of HSP 70 protein and day of incubation was determined by polynomial regression and Spearman rank-order correlation. Statistical analyses were performed using Sigma-Stat 2.03 (SPSS Science Software Ltd.).

RESULTS

Chicken Embryo Survival Results

The embryo mortality was 27% of injected eggs at the control and 20 to 28% at tu2, pa2 and ch10 strains while 53 to 60% at ch5, ch24, and ch25 strains ($P < 0.01$). The vast majority of ones (71–100% of all death embryos) were dying within the first 72 hours after treatment (Table 2).

HSP70 Protein Expression in Embryo and Chick Tissues

The level of HSP70 protein in the liver of chicken embryos from the control group was between E13 and E16 at a similar level from 7899 a.u. (in E15) to 10140 a.u. (in E13). A significant increase in the HSP70 level to the values of 13113 a.u. and 11874 a.u. was observed in E17 and E18, respectively (Figure 1). The above changes can be described by a regression equation:

$y = -294.43x^3 + 14,170x^2 - 224,936x + 10^6$ for $R^2 = 0.454$, where y – level of HSP 70 protein, x – day of incubation. Compared to the control group, embryos infected with *S. aureus* bacteria were characterized by a higher level of HSP proteins (for the entire period between E13 and E19) from 12.3% for the ch10 strain to 19.7% for the

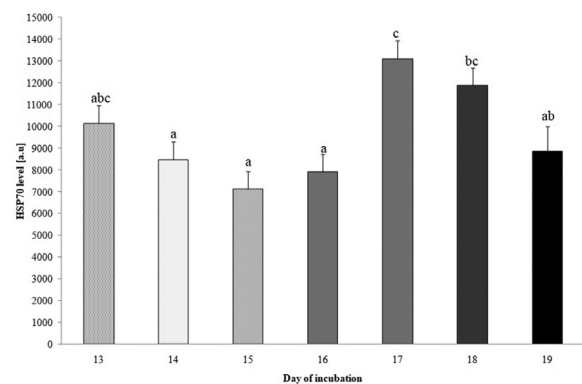


Figure 1. The level of HSP70 (mean \pm SEM) in the liver of chicken embryos from the control group on the following days of incubation. ^{abc-} values marked with different letters differ statistically significantly ($P \leq 0.05$).

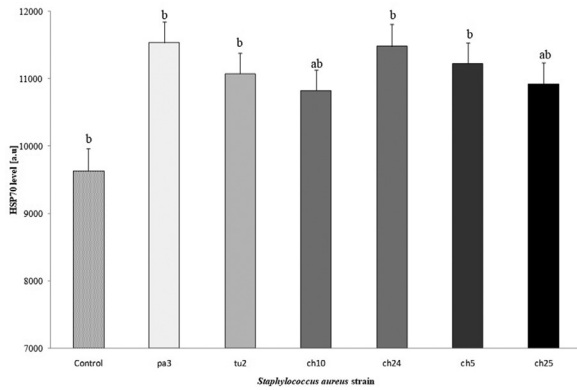


Figure 2. The average level of HSP70 (mean \pm SEM) in the liver of chicken embryos infected with selected strains of *Staphylococcus aureus* in the period from 13 to 19 day of incubation. ^{abc}- values marked with different letters differ statistically significantly ($P \leq 0.05$).

ch5 strain ($P \leq 0.05$; Figure 2). Moreover, on the one hand, changes in the level of these proteins in the liver of a chicken embryo in the following days of incubation were in line with the pattern observed in the control group, and on the other hand, it was noted that the increase in the level of HSP proteins in the groups infected with *S. aureus* coincided with cases of embryo mortality (Table 3).

During the experiment, there were cases of deaths of embryos between E14-E19, which coincided with a jump in the level of HSP70. In the case of the group infected with the tu2 strain, on day 15 of incubation, the loss of 4 chicken embryos was found, which coincided with an increase in the level of HSP70 from 10565 a.u. (E14) to 13580 a.u. (E15), similarly in the group injected with the ch24 strain, where on day 15 the loss of 2 embryos was found, which also in this case increased the level of the tested protein from 9722 a.u. (E14) to 14387.22 a.u. (E15).

Results of the Morphological Analysis of the Blood of Chicken Embryos and Chicks

During the analysis of blood smears of the control group embryos, isolated cases of damaged or destroyed erythrocytes were found, most probably as a result of mechanical damage during preparation of the preparations (Figure 3A). However, in the blood of embryos infected with *S. aureus*, numerous changes were found in the blood count in the form of: lysed red blood cells

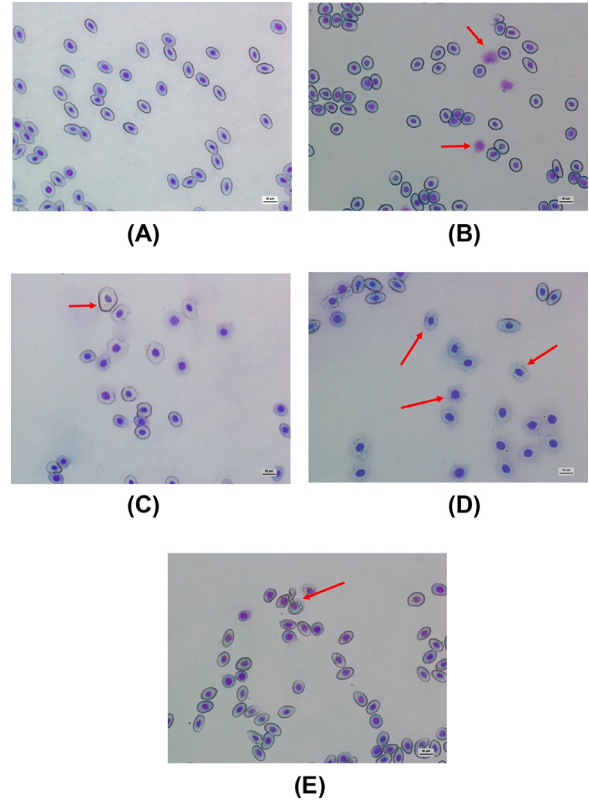


Figure 3. The microscopic view (60 \times magnification) of blood smear of a chick embryo at 14th day of incubation. The eggs were in ovo injected by selected *Staphylococcus aureus* strains at 10th day of incubation; (A) the normal red blood cells of the control group; (B) lysed erythrocyte in the group infected by ch24 strain; (C) the swollen erythrocytes in the group infected by pa3 strain; (D) damaged cell membranes of erythrocytes in the group infected by ch5 strain; (E) changes of erythrocyte morphology in the group infected by tu2 strain.

(Figure 3B); erythrocyte swelling (Figure 3C), damage to erythrocyte cell membranes (Figure 3D) and morphological changes of erythrocytes (Figure 3E).

DISCUSSION

The obtained results of mortality of chicken embryos in the first 72 hours after injection of suspensions containing various strains of *S. aureus* confirmed the differences in the degree of virulence of individual strains previously described by Polakowska et al. (2012). Tu2, ch10 and pa3 strains, showing low virulence for chicken

Table 3. Level (mean \pm SE) of HSP70 [a.u.], between 13 and 19 days of incubation, in the liver of chicken embryos *in ovo* injected within selected *Staphylococcus aureus* strains on day 10th of incubation.

Day of incubation	Strain						
	Control	pa3	tu2	ch10	ch24	ch5	ch25
13	10,139 \pm 2,784 ^{ab}	8,919 \pm 511 ^b	8262 \pm 1,056 ^b	10,707 \pm 572 ^{ab}	10,629 \pm 1,564 ^{ab}	12,345 \pm 3,825 ^a	8,821 \pm 694 ^b
14	8,465 \pm 803 ^a	11,369 \pm 2,141 ^a	10,565 \pm 845 ^a	9,492 \pm 2,498 ^a	9,722 \pm 20 ^a	9,019 \pm 1,370 ^a	9,976 \pm 569 ^a
15	7,114 \pm 2,695 ^d	10,736 \pm 573 ^c	13,580 \pm 680 ^{ab}	11,139 \pm 5,991 ^{abc}	14,387 \pm 286 ^a	7,869 \pm 1,663 ^{cd}	7,859 \pm 2,104 ^{cd}
16	7,898 \pm 208 ^{bc}	13,747 \pm 907 ^a	7210 \pm 495 ^c	8,586 \pm 661 ^{bc}	10,051 \pm 1,341 ^{bc}	8,770 \pm 298 ^{ab}	10,675 \pm 490 ^{ab}
17	13,113 \pm 1,855 ^a	8726 \pm 2,976 ^b	12,567 \pm 1,969 ^a	12,101 \pm 685 ^{ab}	10,187 \pm 2,153 ^{ab}	11,231 \pm 2,675 ^{ab}	10,744 \pm 1,861 ^{ab}
18	11,873 \pm 504 ^b	12,077 \pm 3,023 ^b	15,026 \pm 1,576 ^{ab}	11,933 \pm 1,295 ^b	11,980 \pm 34 ^b	16,781 \pm 2,274 ^a	13,226 \pm 2,954 ^b
19	8,833 \pm 340 ^b	15,136 \pm 692 ^a	10,281 \pm 1,294 ^b	11,801 \pm 777 ^{ab}	13,402 \pm 925 ^{ab}	12,543 \pm 436 ^{ab}	15,159 \pm 54 ^a

^{a-d}Values in the rows marked with different letters differ statistically significantly ($P \leq 0.05$).

embryos so far, caused embryo mortality at the level of 24%, 30% and 32%, respectively. The ch5 and ch25 strains, which were highly virulent for chickens, caused 65% and 60% deaths, respectively, despite being administered in a 10⁸ suspension. The only strain that, contrary to the results obtained in previous studies, caused a high mortality comparable to highly virulent strains was the ch24 strain, which caused a mortality of 60%. Moreover, it is worth noting that such a distribution of mortality probably indicates a rapid growth rate of *Staphylococcus* bacteria after amniotic fluid infection, which results in the fall of the weakest chicken embryos (Polakowska et al., 2012). On the other hand, the observed cases of embryo death in the control group within 72 hours after injection may be the result of disturbed homeostasis caused by manipulation itself (Bruggeman et al., 2005; Lis et al., 2004).

Studies have shown that the presence of HSP 70 is found in the liver of chicken embryos at least from the 10th day of incubation. The level of HSP70 protein in the liver of chicken embryos from the control group remained at a similar level between days 13 and 16. On day 17 of incubation, a significant increase in the level of HSP70 was observed in all study groups. It is probably the result of the initiation of a number of physiological processes leading to the hatching of the chick (van de Ven et al., 2011). The increase in HSP72 protein expression in the liver, but also in the heart and spleen during the subsequent hatching stages was also observed by Lis et al. (2011). During this time, the metabolism of the embryo increases, and with it the concentration of many hormones (Babacanoglu and Yalcin, 2014). This is especially true of thyroid hormones (McNabb, 2006; Sechman et al., 2006) and corticosterone (Rosales, 1994; Veenema et al., 2003). Thyroid hormones: thyroxin (T4) and triiodothyronine (T3) play an important role in the development of many systems of the embryo, e.g., they are responsible for heat production. Their level in the blood plasma of embryos during early embryogenesis is low, however, during the hatching period, when the organism is mobilized, their concentration increases rapidly (McNabb, 2006), as does the concentration of corticosterone (Hayward and Wingfield, 2004; Groothuis et al., 2005).

The change in the level of HSP70 protein in the liver of chicken embryos infected with *S. aureus* in the following days of incubation was in line with the pattern described above, with the provision that it assumed values higher than in the control group. The increase in HSP70 level is probably caused by a strong immune response many antigens derived from *S. aureus* bacteria (Qoronfleh et al., 1993).

Moreover, it appears that the virulence of the staphylococcal strain is positively correlated with the level of HSP70 protein in the liver of the embryos. This hypothesis is supported by the observation that its sharp increase coincided with the period of recorded death of embryos. In this case, an increase in the level of HSP70 in groups infected with tu2 and ch24 strains would indicate the activation of the immune system of the infected

organism in response to the stress factor, which in this case is infection with *S. aureus* bacteria (Srivastava et al., 2011). This is in line with the results of Xu et al. (2014), who observed a change in HSP27 and HSP70 levels in the spleen of chickens under heat stress conditions after administration of antistress compounds. However, in the case of low virulence strains like ch10, it can be speculated that the effects of the body's fight against infection are effective, resulting in low mortality and a slight increase in the level of HSP70 protein.

As a result of the stress factor acting in the cells, there is an increase in the amount of denatured proteins that bind to heat stress proteins. This results in the detachment of heat shock factor 1 (HSF1) from the cytoplasmic complex of HSP. HSF1 is present in the cytoplasm as an inactive monomer unable to bind to DNA under conditions of the organism's homeostasis, while under stress it is activated by protein kinases Pockley (2003). The released phosphorylated HSF1 monomers, once in the cell nucleus, bind to the DNA sequence in the promoter of the HSP gene, inducing an increase in transcription of these genes. If HSP synthesis is reduced, the number of damaged proteins in the cell increases due to genetic instability caused by mutations and disturbances in the cell cycle (Kazmierczuk and Kiliańska, 2009). Such an increase in HSP levels has been described in humans when infected with pathogens such as: *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, or *Staphylococcus epidermidis* (Qoronfleh et al., 1990, 1993). The results of blood counts correspond to the results described above. The damage to the cytoplasmic membranes of red blood cells, deformation of the shape of erythrocytes and spots after lysed erythrocytes observed in the microscopic image of blood smears seem to be caused by toxins produced by *S. aureus* bacteria. It is known that monomeric toxins of staphylococci have the ability to penetrate the cytoplasmic membranes, and this process takes place in two stages (Stachowiak and Bielecki, 2000). The α -toxin plays a special role here, as it damages the lipid bilayer. It is a cytolytic exotoxin produced by most bacteria of the genus *Staphylococcus* (Alouf, 1976). The α -toxin monomers are attached to the cell membrane surface, and the process is still reversible at this stage. Then, there is a gradual transformation of the hitherto hydrophilic monomers into hydrophobic molecules capable of penetrating the membrane, and the hemolysin, after attaching to cholesterol contained in the cell membrane, polymerizes to form pores. At this stage, the process is irreversible. In the case of a low concentration of toxins, metabolic disorders of the host cells are observed, resulting from a violation of the integrity of the cell membrane. However, when their concentration is high enough, cells die by lysis (Stachowiak and Bielecki, 2000).

To conclude, among the studied *S. aureus* strains, the strains ch5, ch25, and ch24 were characterized by the highest virulence for chicken embryos, pa3 and ch10 lower, and tu2 the lowest. Infection of chicken embryos

with selected strains of bacteria of the genus *Staphylococcus* caused changes in the level of heat stress protein HSP70 in the tissues of chicken embryos. The level of heat stress protein HSP70 increases significantly at the beginning of the hatching process on day 17. Infection of chicken embryos with selected strains of bacteria of the genus *Staphylococcus* causes changes in the haematological image of the blood of chicken embryos. The results obtained in the experiment indicate that changes in the level of HSP70 protein may be a useful indicator of the course of infection with *S. aureus* bacteria. It also seems that the chicken embryo model may constitute a convenient research model in the research on the pathogenesis of diseases caused by bacteria.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

- Alouf, J. E. 1976. Cell membranes and cytolytic bacterial toxins. Pages 220–270 in *The Specificity and Action of Animal, Bacterial and Plant Toxins. Receptors and Recognition*. P. Cuatrecasas, ed. Springer, Boston, MA.
- Andersen, M. L., and L. M. F. Winter. 2019. Animal models in biological and biomedical research - experimental and ethical concerns. *An. Acad. Bras. Cienc.* 91:e20170238.
- Babacanoglu, E., and S. Yalçin. 2014. Hormone-mediated maternal stress affects embryonic development during incubation without adverse effect on chick weight and body composition. *Br. Poult. Sci.* 55:186–193.
- Bonar, E., I. Wójcik, U. Jankowska, S. Kedracka-Krok, M. Bukowski, K. Polakowska, M. Lis, M. Kosecka-Strojek, A. J. Sabat, and G. Dubin. 2016. Identification of secreted exoproteome fingerprints of highly-virulent and non-virulent staphylococcus aureus strains, front. *Cell. Infect. Microbiol.* 6:1–15.
- Bruggeman, V., O. Onagbesan, L. Dumez, Q. Swennen, B. De Ketelaere, and E. Decuypere. 2005. Effects of early prenatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on post-natal reproduction in the laying hen (*Gallus gallus*). *Comp. Biochem. Physiol. C.* 141:349–355.
- Fluit, A. C. 2012. Livestock-associated *Staphylococcus aureus*. *Clin. Microbiol. Infect.* 18:735–744.
- Groothuis, T., W. Muller, N. Von Engelhardt, C. Carere, and C. Eising. 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Rev.* 29:329–352.
- Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.
- Hayward, L., and J. Wingfield. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *Gen. Comp. Endocrinol.* 135:365–371.
- Heaton, C. J., G. R. Gerbig, L. D. Sensius, V. Patel, and T. C. Smith. 2020. *Staphylococcus aureus* epidemiology in wildlife: a systematic review. *Antibiotics (Basel)* 9:89.
- Kantidze, O. L., A. K. Velichko, and S. V. Razin. 2015. Heat stress-induced transcriptional repression. *Biochem. (Moscow)* 80:990–993.
- Kaźmierczuk, A., and Z. Kiliańska. 2009. Plejotropowa aktywność białek szoku cieplnego. [The pleiotropic activity of heat-shock proteins]. *Postepy Hig. Med. Dosw* 63:502–521 (Polish).
- Li, P. 2017. Heat shock proteins in aquaculture disease immunology and stress response of crustaceans. Pages 275–320 in *Heat Shock Proteins in Veterinary Medicine and Sciences*. Heat Shock Proteins. A. Asea and P. Kaur, eds. Springer, Cham, Switzerland.
- Li, Y., S. Yu, G. Gu, G. Chen, Y. Zheng, and J. Jiao. 2017. Polymorphisms of heat shock protein 70 genes (HSPA1A, HSPA1B and HSPA1L) and susceptibility of noise-induced hearing loss in a Chinese population: a case-control study. *PLoS ONE* 12:e0171722.
- Lis, M., J. Homa, T. Wojnar, K. Pawlak, K. Głodek, A. Sechman, B. Płytycz, and J. Niedziółka. 2011. Expression of hsp72 protein in tissues of chicken embryos exposed to hyperthermia and administered with ascorbic acid. Book of Abstracts 5th Combined Workshop Fundamental Physiology and Perinatal Development in Poultry, Wageningen. 71.
- Lis, M., T. Jacek, K. Pawlak, and J. Niedziółka. 2004. Time and kind of in ovo injection affects hatchability of chicks. *Sci. Mess. Lviv. State Acad.* 6:93–100.
- Luo, X., X. Zuo, Y. Zhou, B. Zhang, Y. Shi, M. Liu, K. Wang, D. R. McMillian, and X. Xiao. 2008. Extracellular heat shock protein 70 inhibits tumour necrosis factor-alpha induced proinflammatory mediator production in fibroblast-like synoviocytes. *Arthritis Res. Ther.* 10:R41.
- Macario, A. J., M. Lange, B. K. Ahring, and E. C. De Macario. 1999. Stress genes and proteins in the archaea. *Microbiol. Mol. Biol. Rev.* 63:923–967.
- McNabb, A. 2006. Avian thyroid development and adaptive plasticity. *Gen. Comp. Endocrinol.* 147:93–101.
- Międzobrodzki J., and M. Kosecka-Strojek. 2018. Experimental animal models in evaluation of staphylococcal pathogenicity. Pages 265–279 in *Petto-Man Travelling Staphylococci: A World in Progress*. V. Savini, ed. Elsevier, Cambridge, MA.
- Nair, A. R., O. Degheselle, K. Smeets, E. Van Kerkhove, and A. Cuypers. 2013. Cadmium-induced pathologies: Where is the oxidative balance lost (or not)? *Int. J. Mol. Sci.* 14:6116–6143.
- Pockley, A. 2003. Heat shock proteins as regulators of the immune response. *Lancet* 362:469–476.
- Polakowska, K., M. Lis, W. Helbin, G. Dubin, A. Dubin, J. Niedziółka, J. Miedzobrodzki, and B. Władyska. 2012. The virulence of *Staphylococcus aureus* correlates with strain genotype in a chicken embryo model but not a nematode model. *Microbes. Infect.* 14:1352–1362.
- Qoronfleh, M., U. Streips, and J. Wilkinson. 1990. Basic features of the staphylococcal heat shock response. *Antonie Leeuwenhoek* 58:79–86.
- Qoronfleh, M., W. Weraarchakul, and J. Wilkinson. 1993. Antibodies to a range of *Staphylococcus aureus* and *Escherichia coli* heat shock proteins in sera from patients with *S. aureus* endocarditis. *Infect. Immun.* 61:1567–1570.
- Rabczyński, M., R. Adamic, and J. Olszewska-Roczniak. 2006. Antibodies anti-HSP 60/65 – role in atherogenesis, arteriosclerotic plaque risk factor. *Adv. Clin. Exp. Med.* 15:933–939.
- Ramaglia, V., G. M. Harapa, N. White, and L. T. Buck. 2004. Bacterial infection and tissue-specific Hsp72, -73 and -90 expression in western painted turtles. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 138:139–148.
- Rosales, A. 1994. Managing stress in broiler breeders: a review. *Poult. Sci.* 3:199–207.
- Sanden, G., A. Ljungh, T. Wadstrom, G. Banck, J. Miedzobrodzki, and P. Svedman. 1989. Staphylococcal wound infection in the pig: part II. Inoculation, quantification of bacteria, and reproducibility. *Ann. Plast. Surg.* 23:219–223.
- Scanes, C. G., and F. McNabb. 2003. Avian models for research in toxicology and endocrine disruption. *Avian Poult. Biol. Rev.* 14:21–52.
- Sechman, A., J. Niedziółka, M. Lis, and J. Rząsa. 2006. Changes in thyroid hormone levels in chicken embryos exposed to extremely low frequency electromagnetic field. *Archiv. Fur. Geflugelkunde* 70:41–47.
- Soltész, L. V., and P. A. Mardh. 1986. Lethal effect of *Staphylococcus aureus* in embryonated hen's eggs inhibited by coagulase-negative Staphylococci. Pages 75–83 in *Coagulase-Negative Staphylococci*. P. A. Mardh and K. H. Schleifer, eds. Almqvist & Wiksell International, Stockholm, Sweden.

- Srivastava, P., H. Ja, and H. Vardhan. 2011. Chlamydia pneumoniae heat shock protein 60 is associated with apoptotic signaling pathway in human atheromatous plaques of coronary artery disease patients. *J. Cardiol.* 58:216–225.
- Stachowiak, R., and J. Bielecki. 2000. Hemolizyny bakteryjne. [Bacterial hemolysins]. *Postęp. Mikrobiol.* 39:253–270 (Polish).
- Surewaard, B. G. J., A. Thanabalasuriar, Z. Zeng, C. Tkaczyk, T. S. Cohen, B. W. Bardoel, S. K. Jorch, C. Deppermann, J. Bubeck Wardenburg, R. P. Davis, C. N. Jenne, K. C. Stover, B. R. Sellman, and P. Kubes. 2018. α -toxin induces platelet aggregation and liver injury during *Staphylococcus aureus* sepsis. *Cell. Host. Microbe.* 24:271–284.e3.
- van de Ven, L., L. Baller, A. van Wagenberg, B. Kemp, and H. van den Brand. 2011. Effects of egg position during late incubation on hatching parameters and chick quality. *Poult. Sci.* 90:2342–2347.
- van Leeuwen, W., M. Sijmons, J. Sluijs, H. Verbrugh, and A. van Belkum. 1996. On the nature and use of randomly amplified DNA from *Staphylococcus aureus*. *J. Clin. Microbiol.* 34:2770–2777.
- van Leeuwen, W., M. Sijmons, J. Sluijs, H. Verbrugh, and A. van Belkum. 1996. On the nature and use of randomly amplified DNA from *Staphylococcus aureus*. *J. Clin. Microbiol.* 34:2770–2777.
- Veenema, A., O. Meijer, E. Ronald De Kloet, J. Koolahaas, and B. Bohus. 2003. Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. *Horm. Behav.* 43:197–204.
- Wyżewski, Z., K. P. Gregorczyk, L. Szulc-Dąbrowska, J. Struzik, J. Szczepanowska, and M. Niemiałtowski. 2014. Współdziałanie białek szoku cieplnego w organizowaniu struktury przestrzennej białek [Cooperation between heat shock proteins in organizing of proteins spatial structure]. *Postępy Hig. Med. Dosw.* 68:793–807 (Polish).
- Xu, D., W. Li, Y. Huang, J. He, and Y. Tian. 2014. The effect of selenium and polysaccharide of *Atractylodes macrocephala* Koidz. (PAMK) on immune response in chicken spleen under heat stress. *Biol. Trace. Elem. Res.* 160:232–237.