DETERMINATION OF SALMONELLA ANTIBODY TITERS IN THE MEAT OF HEALTHY AND PNEUMONIA DISEASED PIGS

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ABSTRACT

Salmonella is the most common pathogenic microorganism found throughout the environment and common source of bacterial foodborne-related illness. A practical tool for detecting *Salmonella* infection in slaughtered pigs is the serological examination of meat juice. *Salmonella* was determined in meat juices from healthy and pneumonia diseased pigs. *Salmonella* antibodies in pig meat juice were determined by a screening immunoenzymatic analysis method using the IDEXX HerdChek Swine *Salmonella* Test Kit (IDEXX Laboratories, Switzerland). The samples were evaluated according to the optical density ratio of the test sample and the positive control sample. Meat samples from diaphragm pillars were taken from 277 pigs at slaughter house. A total of 63 samples were serologically positive from all tested pig meat juice samples (22.74 %) (CI95 % 18.2 – 28.0 %). One positive sample was found in the meat juice of healthy pigs, while 25 and 37 samples were found to be seropositive in the diseased pig groups, with S/P of 0.76 ($p < 0.001$) and 0.91 ($p < 0.001$) respectively. In pigs with pathological lung changes 35.23 % seropositive samples of all diseased pigs were found. S/P ratio in control group was 0.08 while in pig with different lung lesions meat juice samples S/P ration reached 0.25 and 0.32 respectively ($p < 0.001$). The lowest S/P value in meat juice samples were found in pigs with moderate lung lesions and were found in 56.88 % meat juice samples, while in pigs with intensive lung lesions meat juice samples S/P ratio was 58.13 % ($p < 0.001$). The highest S/P value was found in pigs with intensive lung lesions and reached 0.77 % of all tested samples.

Keywords: *Salmonella, pigs, meat juice, antibody detection.*

INTRODUCTION

Salmonella is one of the most common pathogenic microorganisms found throughout the environment. The pathogenicity of individual serotypes is usually host-specific, such as *Salmonella dublin* is common in cattle, *Salmonella choleraesuis* in pigs, or *Salmonella typhi* in humans. Most of the known serotypes, such as *Salmonella typhimurium* and *Salmonella enteritidis* are not adapted to their specific hosts and therefore can infect many animal species, including humans (Nielsen *et al*., 1995; Evangelopoulou *et al*., 2014; Bonardi, 2017; Sun *et al*., 2020; D'Incau *et al*., 2021).

Salmonella transmission between hosts occur by air or direct contact. Weaned piglets are most often infected with *Salmonella*. It has been found, that *Salmonella typhimurium* can cause disease of young piglets, 6-12 weeks of age, and in some cases of adult pigs, although *Salmonella choleraesuis* can cause salmonellosis in pigs of all ages (Wilcock and Schwartz, 1992; Savic *et al*., 2021). *Salmonella* infection is not uncommon in suckling piglets, although it is extremely rare due to maternal immunity (Gray and Fedorka-Cray *et al*., 2000; Matiasovic *et al*., 2013). Clinically, two porcine salmonellosis syndromes are distinguished, one involving *Salmonella typhimurium* infection with enterocolitis and the other with *Salmonella choleraesuis* infection with septicemia (Wilcock and Schwartz, 1992; Savic *et al*., 2021).

Pigs can be infected with *Salmonella* in their housing, by air during transportation from other diseased pigs or by direct contact from a *Salmonella* contaminated environment (Swanenburg *et al*., 2001; Arguello *et al*., 2013). *Salmonella* is usually isolated from the tonsils, intestinal lymph nodes and intestinal contents of diseased pigs (Proux *et al*., 2000; Swanenburg *et al*., 2001; Novak *et al*., 2007; Deane *et al*., 2022).

Slaughtered pigs, infected with *Salmonella* but showing no signs of the disease, may be the most important source of *Salmonella typhimurium* infection, which poses a threat to the entire food chain. Despite the fact that, based on the consistent application of *post-mortem* inspection rules, classical zoonoses have been largely eliminated and the health of slaughtered animals has improved in recent decades, and the groups of slaughtered animals are more homogeneous, the problem of consumer safety when supplying meat from slaughtered pigs for food has not yet been resolved. Zoonoses occurring during this period are more subclinical, there are no visible changes in carcasses and organs, so, apart from obvious pathological changes, zoonoses in slaughtered pigs are the main cause of diarrhea (food poisoning) in humans (Bonardi, 2017). Slaughtered infected pigs without clinical signs can be the most important source of *Salmonella typhimurium*, which is very dangerous for the entire food chain. The development of effective, inexpensive and rapid diagnostic methods is one of the factors that reduce salmonellosis outbreaks in humans and animals. Since 1993 in Denmark, enzyme-linked immunosorbent assay (ELISA) has been introduced in salmonellosis monitoring studies. The tests were carried out using blood serum from pigs, and meat juice was used for slaughtering pigs (Nielsen, 1998; Mousing *et al*., 1997; Bak and Sørensen, 2006).

ELISA in experimental studies and routine diagnostics shows important advantages - specificity, sensitivity and easy application (Gray *et al*., 1999; Nowak *et al*., 2007). Considering the fact that antibody titers in meat juice are slightly lower compared to blood serum, the advantage of meat juice testing by changing the sample dilution ratio has been proven, especially since it is inexpensive and relatively fast compared to bacteriological test methods (Proux *et al*., 2000; Viana *et al*., 2020).

MATERIALS AND METHODS
Selection and grouping of pigs for study
For the detailed analysis of lung diseases in pigs, detected during *post-mortem* inspection, one meat company was selected, to which pigs raised in different regions of Lithuania were transported for slaughter. For further research, pigs were reared under the same conditions of rearing and storage and transported to the slaughterhouse at a distance of 65 km. After slaughtering, the internal organs of the pigs were examined and the macroscopic pathological changes of the lungs, heart and liver were registered. Based on the methodology for the assessment of lung pathological changes proposed by Blaha and Neubrand (1994), according to the determined data of lung changes, groups of pigs were formed: pigs with moderate pneumonia (11-30 % of lungs affected) with signs of mild pleuritis (group Pn 2 + Pl 2) and pigs with intensive pneumonia (over 30% of lungs affected) with pleuritis, covering a large area of the lungs (group Pn 3 + Pl 3). It should be noted that all lung changes identified had signs of a chronic type. The control group consisted of pigs that did not show any pathological changes during post-*mortem* examination.

Collection of muscle samples and extraction of meat juice
At the slaughterhouse after 1 hour after slaughter *musculus pillar diaphragmaticus* in size of 3.0 x 1.0 x 1.0 cm were collected from slaughter pigs. The muscle samples were placed in 50 ml plastic bags and transported to the laboratory immediately after collection. In total, muscles were collected from 277 pigs. Muscles were frozen at 220 °C. 1 day before the beginning of the study, the investigated meat samples were transferred to a refrigerator at a temperature of +4 °C and kept for 24 hours. During thawing, meat juice was released from the muscles and collected in the bottom of the containers.

Detection of *Salmonella* antibodies in meat juice samples
Antibodies against *Salmonella* spp. in pig meat juices were determined by the overview method of immunoenzymatic analysis. A commercial diagnostic kit (IDEXX HerdChek Swine *Salmonella*, IDEXX Laboratories, Switzerland) was used for this purpose. Analysis was performed consistently according to the diagnostic kit manufacturer's methodology. Each of the 96 wells of the IFA plate was coated with antigens (serogroups B, C1, D). Before the study, the meat juice of the test pigs was diluted in a ratio of 1:2. Diluted meat juice samples were

transferred into the wells of the plastic plate intended for analysis with a multichannel pipette, 100 µl each. Undiluted negative and positive control serum samples of 100 µl each were also added to the wells of the plate intended for them. The samples were incubated for 30 min. at a temperature of 25 **°C**. After incubation, the wells of the plate were washed with a buffer solution of 300 µl to each well of the plate. The procedure is repeated 5 times for 2 minutes. After incubation, the plate was coated with 100 µl of specific immunoglobulin conjugated with horseradish peroxidase. Incubated for 30 min. at a temperature of 25 **°C**. The plate was washed again with buffer solution 300 µl to each well of the plate, repeating the procedure 5 times. After washing, 100 µl of tetramethylbenzidine substrate was added to each well of the plate and incubated for 15 min. at room temperature. The reaction was stopped by adding 100 µl stop reagent (hydrochloric acid) to the wells of the microplate.

The intensity of the color is directly proportional to the amount of antibodies in the test samples. The results of the research were evaluated by spectrophotometer by measuring the optical density (OD) of the test sample at a wavelength of $\lambda = 650$ nm. The difference between the means of the positive and negative control sera had to be greater than or equal to 0.150 when evaluating the results of the assay. The mean of the negative control serum had to be less than or equal to 0.200. The OD of the samples was compared with the positive control OD of the buffer solution. Samples are considered positive if their OD is 10 percent or more high than the OD value of the buffer positive control or ratio of optical density of the test sample and the positive control sample $S/P \geq 0.25$. Samples are negative if their OD values are less than 10% buffer control OD or $S/P < 0.25$. To determine the reliability of the test results, the ELISA was repeated 2 times (S/P-1, S/P-2).

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics Version 27. Differences in the test properties of the compared groups are expressed as means and RMSE (root mean square errors). The differences between the investigated groups were evaluated using Fisher's LSD criterion ($\alpha=5\%$). The differences were considered to be statistically significant when $p < 0.05$.

CI was calculated by the Wilson method.

RESULTS AND DISCUSSION

Meat juices from 277 slaughtered pigs were tested for *Salmonella* specific antibodies. Evaluating the results of the analysis of pig meat juice using the ELISA method, it was found that 63 pig meat juice samples (22.74 %) were serologically positive of all tested samples (CI 95 % 18.2 – 28.0 %). Analyzing *Salmonella* antibody titers, the number of seropositive pigs detected at *post-mortem* examination was unevenly distributed according to the degree of lung damage in pigs. *Salmonella* antibodies were found in only one sample of all tested meat juice samples of the control group of pigs, while 35.23 % of seropositive samples were found in the meat juice samples of pigs with pathological lung changes of all samples from diseased pigs. 22.90 % of pigs with moderate lung lesions meat juice

samples were serologically positive, and 28.70 % with intensive lung lesions meat juice samples were serologically positive.

To determine the reliability of the test results, the ELISA was repeated 2 times. The results of ELISA tests are presented in Table 1, where S/P-1 is the result determined for the first time, and S/P-2 is the result for the second time. S/P was 0.08 ($p < 0.001$) in meat juice from control pigs, while S/P in meat juice from diseased pigs was greater than 0.25 of all tested samples. S/P in meat samples of pigs with intensive lung lesions was 0.32 ($p < 0.001$). The results obtained in both study periods did not differ and confirmed their reliability.

Table 1. Results of the *Salmonella* specific antibody test in the tested meat juice samples.

	Control group; n = 38	Pn 2 + Pl 2; n = 109	Pn 3 + Pl 3; n = 129
S/P–1	0.08 ± 0.008^a	0.26 ± 0.041^b	0.32 ± 0.047^b
S/P–2	0.08 ± 0.006^a	0.25 ± 0.041^b	0.32 ± 0.05^b
a, b – $p < 0.001$			

In the evaluation of pig lung pathologies and *Salmonella* antibody titers in meat juice, it was found that one sample of the control group was serologically positive, while 25 and 37 samples were found to be seropositive in the diseased pig groups, with S/P of 0.76 ($p < 0.001$) and 0.91 ($p < 0.001$) respectively (Table 2).

Table 2. Effect of lung lesions on *Salmonella* antibody test results.

Group	Result	n	S/P–1	S/P–2
Control group	Seronegative	38	0.08 ± 0.006	0.07 ± 0.003
	Seropositive	1	0.29	0.29
Pn 2 + Pl 2	Seronegative	84	0.12 ± 0.024^a	0.11 ± 0.024^a
	Seropositive	25	0.76 ± 0.112^b	0.71 ± 0.119^b
Pn 3 + Pl 3	Seronegative	92	0.08 ± 0.003^a	0.07 ± 0.003^a
	Seropositive	37	0.91 ± 0.116^b	0.93 ± 0.127^b
Pn 2 + Pl 2 Pn 3 + Pl 3	Seronegative	214	0.09 ± 0.009^a	0.09 ± 0.01^a
	Seropositive	63	0.84 ± 0.082^b	0.83 ± 0.089^b
a, b – $p < 0.001$				

When evaluating the distribution of S/P in the meat juice samples of healthy and diseased pigs, it was found that the lowest S/P value (0.10) was in 79.48 % meat samples of the control group of all tested samples and only 5.12 % amounted to the highest level (0.30). The lowest S/P value of diseased pigs was 57.56 % while the highest value was 3.00 and reached 0.42 % (Fig. 1).
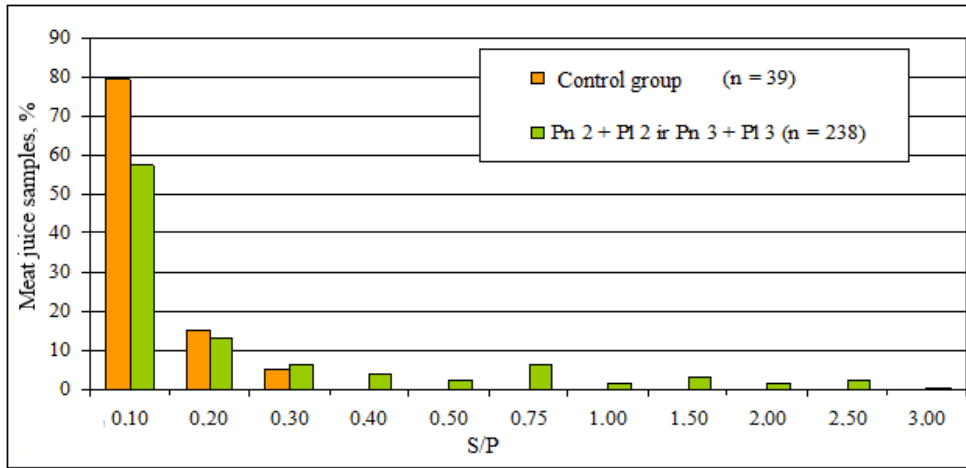
Fig. 1. Optical density ratio (S/P) of *Salmonella* specific antibodies in meat juice of healthy and diseased pigs.

When analyzing the distribution of *Salmonella* antibodies S/P values according to the degree of lung damage, the values of Pn 2 + Pl 2 and Pn 3 + Pl 3 groups were almost evenly distributed - the lowest S/P value in meat juice was found in pigs with moderate lung lesions and was found in 56.88 % meat juice samples, while in pigs with intensive lung lesions meat juice samples S/P ratio was 58.13 %. In addition, the highest value (3.00) was found in pigs with intensive lung lesions and reached 0.77 % of all tested meat juice samples in this group. The highest S/P value of Pn 2 + Pl 2 group samples was 2.5 and reached to 1.83 % of all tested meat juice samples in this group (Fig. 2).
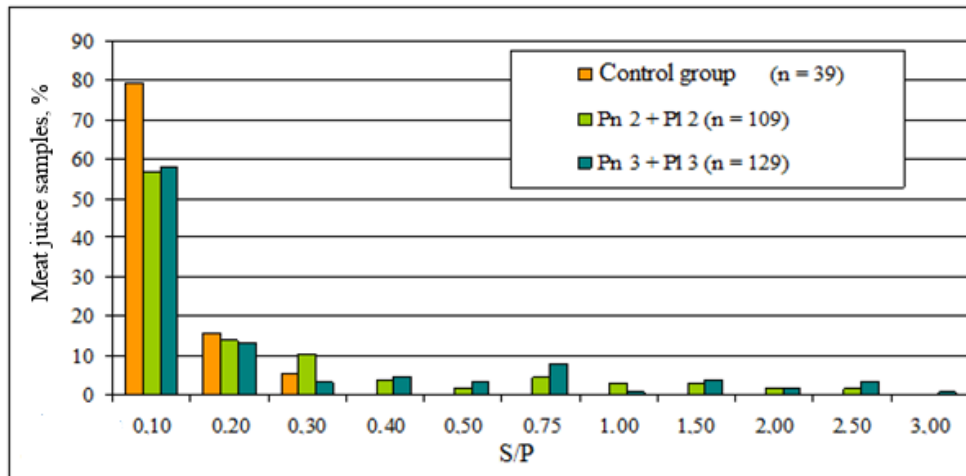

Fig. 2. Optical density ratio (S/P) of *Salmonella* specific antibodies in meat juices of different pig group.

Blood serum is mostly used in biochemical and serological tests. The use of blood serum in routine *post-mortem* examination is difficult due to the speed of work in the slaughter line and the too short time for taking blood for examination. Therefore, in the search for newer and better test methods to improve the quality of *post-mortem* inspection, the suitability of meat juices has been proven due to their simple use and the possibility of automating the test process itself (Nielsen, 1998). The collection of blood samples on the farm as part of the ante-mortem inspection can complicate the slaughter process due to the labor and time costs of sampling. And the method of taking meat samples after slaughtering the animal turned out to be superior also because it did not require specific technical knowledge. In addition, the testing used to identify the health status of pigs can be not only improved but also simplified by implementing centralized testing systems (Le Potier *et al*., 1998). Pig salmonellosis can cause not only economic losses, but also a lot of damage to human health, so very fast, accurate and sensitive methods for S*almonella* detection are needed. Routine salmonellosis testing introduced in Denmark has proven to be effective in reducing salmonellosis outbreaks in the country (Wegener *et al*., 2003). Our immunoenzymatic analysis using meat juices suggested that this ELISA method could be applied as a rapid method for *Salmonella* spp. as a routine *post-mortem* monitoring method. It should also be noted that the ELISA method only confirmed positive samples in the meat juice at the genus level, but did not identify the species or serotype. On the other hand, identification of the species or serotype of *Salmonella* is not very important for epidemiological studies or monitoring purposes, especially in pig farms where *Salmonella* infection is very common and where more than one *Salmonella* serotype has been isolated (Feder *et al*., 2001; Novak *et al*., 2007; Edel *et al*., 2023).

CONCLUSION

The results of our study showed that 22.74 % of all tested samples of pig meat juice were serologically positive. The number of seropositive pigs was unevenly distributed according to the degree of lung damage in the pigs determined at *post-mortem* examination. *Salmonella* antibodies were found in only one sample of all tested meat juice samples of the control group of pigs, while 35.23 % of seropositive samples were found in the meat juice samples of pigs with pathological lung changes of all samples from diseased pigs. 22.90 % were serologically positive for *Salmonella* in meat juice samples of pigs with moderate lung damage, and 28.70 % of pigs with severe lung damage.

The obtained results allow this ELISA method to be applied to the control of food products, when it is necessary to check the suspicious raw material very quickly, within one day.

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