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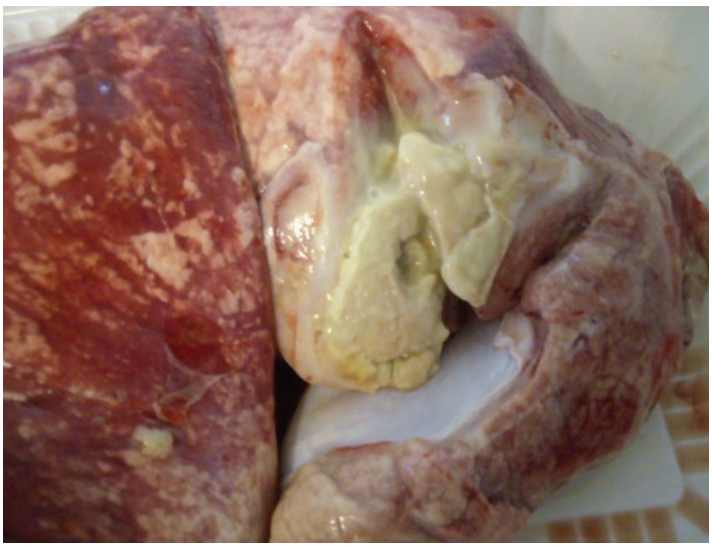

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Original Research Article

GENOTYPIC CHARACTERIZATION OF A *Trueperella pyogenes* STRAIN AS A MAJOR CAUSATIVE AGENT OF METRITIS, ABORTION AND DEATH IN *Bubalus bubalis*

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Abstract: *Trueperella pyogenes* is a species of commensal bacteria which is present on the upper respiratory, urogenital and gastrointestinal mucosae of cattle. This species is able to cause pyogenic infections and health risks, alone or in association with other pyogenic bacteria. However, systemic disease with abortion and death in water buffalo has not yet been documented. Here, we isolated a strain of *T. pyogenes*, from a pregnant water buffalo (*Bubalus bubalis*) with metritis and pneumonia, which finally caused abortion and death in the affected host. Thereafter, the virulence genes and antibiotic resistance of the isolate were investigated. Single PCR method confirmed the presence of the well-known virulence genes of *T. pyogenes* including *plo, nanH, nanP, cbpA, fimA, fimC, fimE* and *fimG* genes. Antibiotic susceptibility test revealed that this isolate was resistant against Tetracycline, Erythromycin and Trimethoprim sulfamethoxazole. Furthermore, using Box-PCR method, it was determined that DNA fingerprint pattern of this isolate was different from that of a control strain (*T. pyogenes* ATCC 19411). The results of the present study indicated that *T. pyogenes* can cause a systemic lethal disease in water buffalo. However, it seems that host and environmental conditions may also contribute to such infection. To our knowledge, this is the first report of a buffalo with pneumonia, metritis, abortion and death caused by *T. pyogenes*.

Key words: *Trueperella pyogenes*; *Bubalus bubalis*; buffalo; BOX PCR; abortion, death

Introduction

Trueperella pyogenes which is formerly known as *Actinomyces pyogenes* and *Arcanobacterium pyogenes*, has recently been reclassified based on distinctive 16S rRNA gene sequences. This bacterium is an irregular, non-motile, non-spore-forming, aerobic, commensal, Gram-positive coccobacillus which is normally isolated from the upper respiratory, urogenital and gastrointestinal tracts (1, 2, 3). It is proposed as a worldwide distributed secondary pathogen which may cause disease conditions such as acute and summer mastitis, metritis, clinical and subclinical endometritis, cutaneous and visceral abscesses, arthritis, pneumonia, endocarditis, osteomyelitis and several other suppurative infectious diseases in a broad range of domestic and wild animals including cattle, swine, sheep, goat, camel, buffalo, deer, antelope, reptiles and also birds (3, 4). *T. pyogenes* is closely associated with other pyogenic bacteria such as *E.coli*, *Streptococcus dysgalactiae* and *Fusobacterium necrophorum* and this can increase its constant presence in pyogenic bacterial infections and resistance

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against antimicrobial agents (5). *T. pyogenes* not only causes pyogenic infections and health risks, mostly in cattle, but also greatly affects economy of animal husbandry by drastic reduction in lactation and fertility of cattle which results in culling of these animals or removal of involved organs, mainly liver and lung, at slaughter houses (6). Moreover, its probable pathogenicity for human, especially in immunosuppressed and diabetic patients, and the role of consumption of dairy products in public health, further highlights the importance of *T. pyogenes* (7, 8).

Several pathogenic properties are known in *T. pyogenes* which can increase its pathogenicity (2, 3). Pyolysin (*plo*), as one of its major virulence factors, causes hemolysis and cytolysis of leukocytes. This hemolysin attaches to the cell membrane cholesterol and lyses the cells by generating pores in the cell membrane. It also plays a role in cytokines expression and tissue damages. On the other hand, bacterial adhesion to epithelial cells, colonization as well as degradation of DNA and sialic acid, are attributed to H and P neuraminidases (*nanH* and *nanP*) of this bacterium. Furthermore, these neuraminidases block phagocytosis of bacterial cells by increasing membrane viscosity (2). Different types of fimbriae are expressed by *T. pyogenes* including A, G, E and C (9). These types of fimbriae are required for adherence to membranes and epithelial cells. Collagen and fibronectin-binding proteins (*Cbp*, *Fbp*) are essential for adhesion to collagen-rich tissues (types 1, 2, 4) and fibronectins. In addition, the protease and DNase of *T. pyogenes* provides nutrients for the bacteria through degradation of proteins and nucleic acids (3).

Antibiotic therapy is a common treatment in metritis. Consequently, overuse of antibiotics as treatment or as preventive and growth inhibitor agents has caused development of resistance to several antibiotics in *T. pyogenes* (10, 11, 12). Therefore, antimicrobial susceptibility tests along with molecular determination of antibiotic resistance genes can facilitate selecting appropriate antibiotics. The aim of this study was to determine the virulence characterizations and antibiotic resistance genes in a primary pathogenic *T. pyogenes* strain, causing abortion and death in a water buffalo (*Bubalus bubalis*), and determining whether bacterial colonies isolated from aborted fetus and different organs of infected *B. bubalis* are identical. This study was the first of its kind carried out in Iran to examine these specific virulence and antibiotic resistance genes.

Materials and methods

Sampling

Sampling was conducted at multiple times during June 2017 from a pregnant buffalo with metritis and abundant pyogenic discharges. Clinical signs including tachycardia, tachypnea, anorexia, fever and weakness were observed in the animal. Abortion occurred two days after the first sampling; hence, a set of samples was also collected from cotyledons, abomasum and fetal fluids. Necropsy was performed one day later, since the buffalo died from the disease. Extensive abscesses were observed in internal organs, particularly in liver and lung (Figure 1). All samples were collected in individual sterile containers, in ice packed coolers, and sent to the bacteriology laboratory for further culturing and identification of potential infectious agents.

Bacterial isolation and growth conditions

Following preparation of smears from all specimens, they were cultured on blood agar supplemented with 5% sheep blood and MacConkey agar (Merck, Germany) and incubated at 37 °C for 48 h under 5-10% CO_2. Gram-stained smears of all plated isolates were also prepared. To identify the bacteria, several biochemical tests including catalase, oxidase, urease and CAMP tests, nitrate reduction, gelatin hydrolysis, esculin hydrolysis, litmus milk, pitting of Loeffler serum, and fermentation of glucose, lactose, maltose, mannitol, sucrose and xylose were performed on isolates (13).

Antimicrobial susceptibility test

Antibiotic sensitivity was tested by Kirby-Bauer disk diffusion method. Susceptibility of samples was tested against routine medicine and veterinary medicine antibiotics on Mueller Hinton agar (Merck, Germany) supplemented with 5% sheep blood. All isolates were screened for their resistance profile against Gentamicin (GM 120 µg), Ampicillin (AP 25 µg), Penicillin G (PG 10 units),

Enrofloxacin (ENF 5 µg), Tetracycline (TE 30 µg), Amoxicillin (A 25 µg), Spectinomycin (SPC 100 µg), Trimethoprim sulfamethoxazole (TS 25 µg), Erythromycin (E 15 µg), Ciprofloxacin (CIP 5 µg), Cefalexin (CFX 30 µg), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (14). Results were read after 48-72h of incubation.

DNA extraction and polymerase chain reaction (PCR)

Isolated bacteria were cultured in TSB broth (Merck, Germany) supplemented with 5% bovine serum and incubated at 37 °C for 48 h. Thereafter, 3 ml of TSB broth was centrifuged at 10,000 g for 10 min at 4 °C. The pellet was washed once with saline solution. Finally, genomic DNA was extracted using a commercial DNA extraction kit for Gram-positive bacteria according to the manufacturer's instruction (MBST, Iran). The extracted DNA samples were stored at -20 °C. To identify *T. pyogenes*, a PCR assay was performed which targeted 16S-23S rDNA intergenic spacer region in genomic DNA (15). PCR was done in 25 µl of a reaction mixture containing 12.5 µl of 2X Master mix (BIONEER Cat. No. PCR-106S-CSTM), 0.1 µl of each primer (100 pmol), (F: 5'- GTTTTGCTTGTGATCGTGGTGGTTATGA-3', R: 5'- AAGCAGGCCCACGCGCAGG- 3') (BIONEER, Korea), 3 µl of template DNA and 7.5 µl of distilled water. The reaction was carried out in a thermocycler (TC-512 Techne, England) as follows; an initial denaturation at 95 °C for 10 min, then 30 cycles of 95 °C for 30 sec, 64 °C for 15 sec, 72 °C for 30 sec and a final extension at 72 °C for 7 min. The amplification products (5 µl) were resolved by electrophoresis on 1.5% agarose gel in 1x TBE for 1 h at 100 V. Afterwards, the agarose gel was stained with 1 µg/ml ethidium bromide (CinnaGen, Cat No. MR7721C) and screened using UV-illuminator (BIORAD, UK).

Screening of genes encoding virulence factors

Single PCR method was used to evaluate the presence of known *T. pyogenes* virulence genes including *plo, nanH, nanP, cbpA, fimA, fimC, fimE* and *fimG* in the isolates (16, 5). Each PCR assay was performed in a reaction mixture with the final volume of 20 µl containing 10µl of 2X Master Mix, 0.1 pmol of each of forward and reverse primers (100 pmol), 2 µl of template DNA and 6 µl of distilled water. The sequence of primers and PCR conditions are presented in table 1.

Table 1: Oligonucletide primer sequences and PCR conditions

Virulence factor / Target gene	Primer sequence (5'- 3')	Amplicon size (bp)	Annealing (°c)
Pyolysin *(plo)*	F: TCATCAACAATCCACGAAGAG R: TTGCCTCCAGTTGACGCTTT	150	60
Neuraminidase H *(nanH)*	F: CGCTAGTGCTGTAGCGTTGTTAAGT R: CCGAGGAGTTTTGACTGACTTTGT	781	60
Neuraminidase P *(nanP)*	F: TTGAGCGTACGCAGCTCTTC R: CCACGAAATCGGCCTTATTG	150	60
Collagen-binding protein *(cbpA)*	F: GCAGGGTTGGTGAAAGAGTTTACT R: GCTTGATATAACCTTCAGAATTTGCA	124	60
Type A fimbria *(fimA)*	F: CACTACGCTCACCATTCACAAG R: GCTGTAATCCGCTTTGTCTGTG	605	57
Type G fimbria *(fimG)*	F: ACGCTTCAGAAGGTCACCAGG R: ATCTTGATCTGCCCCCATGCG	929	57
Type E fimbria *(fimE)*	F: GCCCAGGACCGAGAGCCAGGGC R: GCCTTCACAAATAACAGCAACC	775	55
Type C fimbria *(fimC)*	F: TGTCGAAGGTGACGTTCTTCG R: CAAGGTCACCGAGACTGCTGG	843	60

Genotyping

Using BOX-PCR, DNA fingerprints of the isolates were obtained to determine the relationships among them (17, 18). All of the isolates obtained from metritis, cotyledon of the aborted fetus and abscesses in the liver and lung of the buffalo (after necropsy). Genomic DNA of the control strain of *T. pyogenes* and distilled water were used in BOX-PCR as positive and negative controls, respectively. BOX-A1R primer (5′-CTACGGCAAGGCGACGCTGACG- 3') was used for amplification. PCR mixture was prepared using 12.5 µl of 2X Master Mix, 0.2 pmol of the primer (100 pmol/µl), 1 µl of template DNA (100 ng) and 9.5 µl of distilled water in a final volume of 25 µl. The reaction was carried out as follows; an initial denaturation at 95 °C for 2 min, then 34 cycles of 95 °C for 1 min, 53 °C for 1 min 72 °C for 5 min and a final extension at 72 °C for 10 min. The amplification products (5 µl) were resolved by electrophoresis on 1.5% agarose gel for 3 h at 70 V. Afterwards, the agarose gel was stained with ethidium bromide and screened using UV-illuminator. Similarities and differences between amplified fragments were analyzed using NTSYSpc software (version 2.1, USA).

Results

Culturing and biochemical tests

Small, irregular Gram-positive coccobacilli were observed in smears obtained from the organs. After 48 h, small white colonies with fine complete hemolysis were detected on the blood agar plates, but no growth was observed on the MacConkey agar plates. Smears from cultured colonies also contained irregular Gram-positive coccobacilli. Catalase, oxidase, urease, nitrate reduction and esculin tests were negative for all isolates. On the contrary, the results of gelatin hydrolysis and pitting of Loeffler serum were positive. In litmus milk, production of acid, curd, reduction and protein digestion were observed. The isolates fermented lactose, sucrose, xylose and maltose; however, none of them fermented mannitol. CAMP test with *Staphylococcus aureus* was also positive.

Antimicrobial susceptibility test

The results revealed that all of the isolates were sensitive to Ampicillin, Penicillin G, Amoxicillin, Spectinomycin, Cefalexin and Ciprofloxacin. By contrast, they were resistant against Tetracycline, Erythromycin and Trimethoprim sulfamethoxazole. Susceptibility grade of the isolates against Enrofloxacin and Gentamicin was intermediate.

16S-23S rDNA PCR

As shown in figure 2, DNA fragment of expected size (122 bp) was observed for all of the isolates obtained from metritis, cotyledon, liver and lung; therefore, it was confirmed that all of them were *T. pyogenes*. No DNA band was amplified from the negative control.

Genes encoding virulence factors

DNA fragments corresponding to the sequences of investigated virulence genes including *plo, nanH, nanP, cbpA, fimA, fimC, fimE* and *fimG* were successfully amplified from the extracted DNA samples of all isolates. The results of these PCR assays are depicted in figure 3.

Box PCR

Eight different bands of about 300, 550, 600, 650, 700, 800, 1000 and 1200 bp compared with 1 kb standard ladder (CinnaGen Cat No. PR 901645) were observed for all of the isolates (Figure 4). As illustrated in figure 5, an identical DNA fingerprint pattern was detected for all of the isolates, suggesting that they were the same strain. However, this pattern was completely different in comparison to that obtained from the control strain of *T. pyogenes*.

Discussion

Over the past decades, *T. pyogenes* has been known as an opportunist suppurative pathogen in domestic animals (19). A wide range of studies have been done on this microorganism which showed it can affect a large group of hosts and causes multiple diseases in different organs and a geographic region (3). Mastitis, metritis, pyometra, umbilicitis, lymphadenitis, prostatitis,

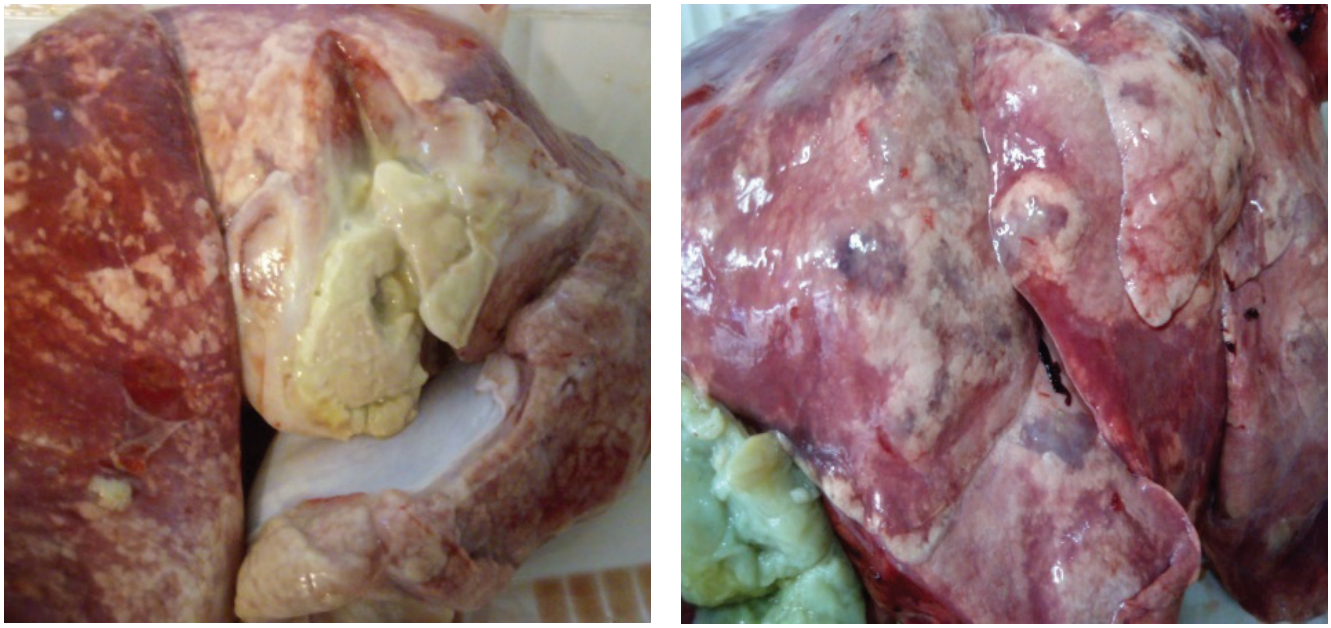

Figure 1: Necropsy findings; diffusive abscesses and pyogenic lesion in liver and lung

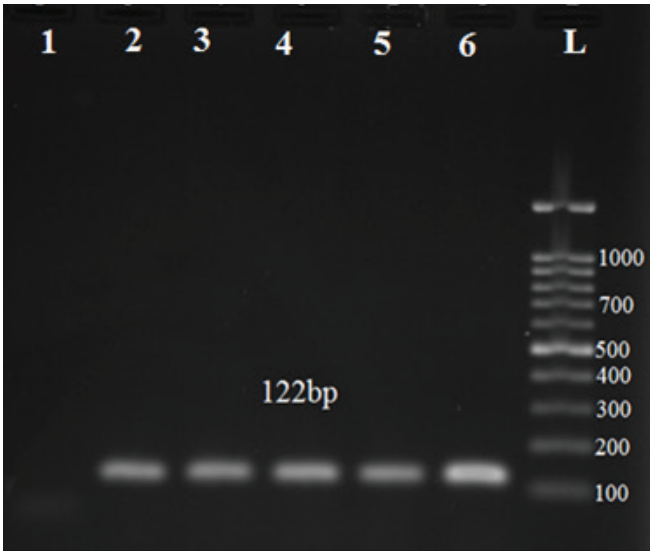

Figure 2: 16S- 23S rDNA- ISR PCR. Lane 1. PCR product using distilled water as negative control. Lane 2-5: PCR product of DNA from bacteria isolates in this study, Lane 6. PCR product of DNA from ATCC 19411 *T. pyogenes* strain as positive control, Lane L. 100 bp ladder DNA marker

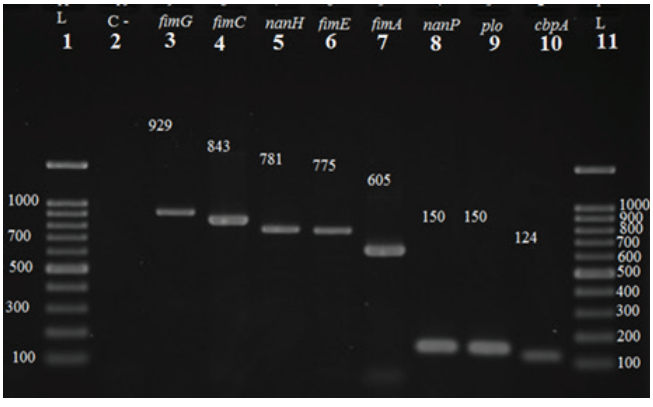

Figure 3: Single PCR for genes encoding virulence factors in *T. pyogenes*. Lane 1: 100 bp ladder DNA marker, Lane 2. PCR product using distilled water as negative control, Lane 3-10. PCR products of DNA from a *T. pyogenes* isolate in this study with specific primers for *plo, nanH, nanP, cbpA, fimA, fimC, fimE and fimG* genes. Lane 11: 1 kb ladder DNA marker

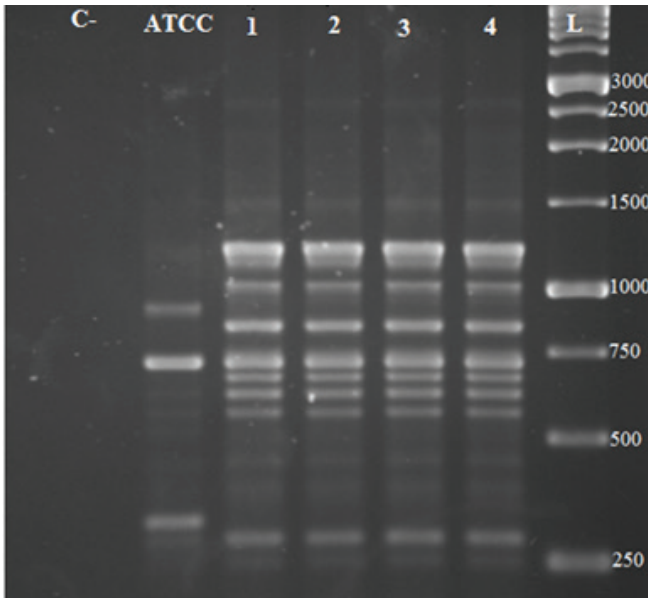

Figure 4: Box-PCR fingerprinting, Eight different bands with molecular weights of 300, 550, 600, 650, 700, 800, 1000, 1100 and 1200 bp compared with 1 kb standard ladder Lane L) were observed in isolates from metritis in the buffalo, cotyledon of aborted fetus and abscesses in liver and lung (Lane 1-4). A *T. pyogenes* strain used as positive control had a different composition of bands (Lane ATCC 19411)

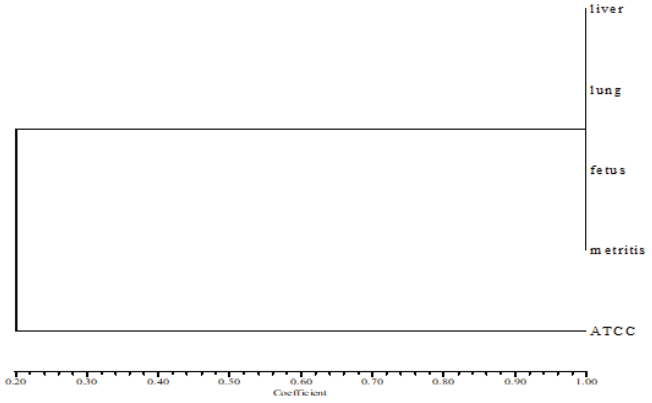

Figure 5: Dendrograms of clonal relationship based on BOX PCR by NTSYSpc (version 2.10e)

orchiditis, pericarditis, encephalitis, septicemia and other pyogenic infections are described as the most important clinical complications caused by *T. pyogenes* in cattle; among which, mastitis, metritis and pneumonia are the most common forms (2, 19). According to literature, pneumonia is a common complication since cold, stress and poor housing conditions underlie this condition in the infected animals (20). The case of infectious pneumonia reported in this study came as a surprise, since pneumonia is reported in ruminants most commonly during warm season. Probably, poor housing conditions, noise from constructions around the stable and pregnancy induced additional stress in the studied animal, and promoted *T. pyogenes* pneumonia. In addition to the effects of these factors, several other elements such as food and water deprivation, mix livestock farming, transport, management and feeding quality have been proposed as the main predisposing factors for *T. pyogenes* infections (21). Clinical implications of pyogenic bacteria such as mastitis and abscess formation in organs have a large impact on the herd economy and result in a huge loss of revenue in livestock. *T. pyogenes* may be responsible for direct economic losses due to condemnation of carcasses or different organs, particularly liver and lung (22, 23, 24, 25, 26).

Metritis caused by *T. pyogenes* after parturition is highly prevalent in cattle. Moreover, other pathogenic bacteria such as *E.coli* and *Fusobacterium necrophorum* commonly increase the severity of this infection (5). However, pure cultures of *T. pyogenes* derived from metritis were documented in this study during the pregnancy and before the parturition. These results indicated that *T. pyogenes*, as a member of gut flora, could also be proposed as a primary pathogen causing diseases in different organs of the host (3). All of the isolates in this study were identified using

morphological and biochemical tests. Although, biochemical and phenotypical tests are typically used to identify *T. pyogenes*, variation in characteristics of this bacterium may cause some difficulties in this process (27, 11). Magdalena et al. identified 14 biotypes of *T. pyogenes*, among which, some showed weak and others, strong hemolytic reactions by CAMP test (28). However, only one biotype was detected and verified by a recent highly valuable molecular method in the present study. Ashrafi Tamai et. al. eight different biotypes were identified among the isolates based on the phenotypical properties such as hemolysis, CAMP and biochemical analysis (11). Although cephalosporins, tetracycline, penicillins and some other beta-lactam antimicrobial agents are considered as the antibiotics of choice for *T. pyogenes* infections, recently antimicrobial resistance against some of these agents have been detected (3). *T. pyogenes* isolates identified in this study were sensitive to ampicillin, penicillin G, amoxicillin, spectinomycin, and ciprofloxacin, whereas they were resistant against tetracycline, erythromycin and trimethoprim sulfamethoxazole. In a similar study, (85.5%) and (9.1%) resistance to tetracycline and erythromycin were reported, respectively (29). In another study, the highest resistance was observed against trimethoprim sulfametoxazol (49.3% and 72.3%) followed by norfloxacin (10.9% and 17%) and tetracycline (9.2% and 10.8%) (20, 11). Indeed, excessive use of antimicrobial agents leads to antibiotic resistance in *T. pyogenes* infections. Besides, intensive administration of tetracycline and macrolides in veterinary medicine as feed additives with preventive or growth inhibitor purposes has resulted in antibiotic resistance of *T. pyogenes* (20, 11). Although some antibiotics are effective against isolates under laboratory conditions, they are not able to diffuse into the center of granulomatous lesions in progressive stages of the disease, which complicates the treatment of *T. pyogenes* infections (19). Therefore, late diagnosis and improper choice of antibiotics are associated with a poor prognosis. Additionally, antibiotic resistance is a great concern in medical administration of antibiotics in humans.

T. pyogenes expresses 8 different virulence genes which play crucial roles in its pathogenic properties (3). In the present study*, plo, nanH, nanP cbpA, fimA, fimC, fimG* and *fimE* virulence genes were detected in all of the isolates. One of the most important virulence genes is pyolysin (*plo*), an extracellular toxin, which is one of the first pathogenic factors detected in *T. pyogenes* (3). Although pyolosin was primarily identified as a hemolysin of red blood cells in a variety of animal species, its cytolytic effect has been demonstrated in several different host cells such as polymorphonuclear leukocytes (PMNS) and macrophages (30, 3). Several studies have reported that all *T. pyogenes* strains encode pyolsin gene but the frequency of its expression is higher in pathogenic strains involved in clinical complications (31, 32, 33). Neuraminidases, encoding by *nanH* and *nanP* genes, are two other main virulence factors of *T. pyogenes*. Neuraminidase or sialidase is an extracellular enzyme which cleaves sialic acid residue from carbohydrates and glycoproteins to be used as a carbon source of energy by bacteria (34). Neuraminidase has a major role in adhesion of bacteria to the epithelial cells, particularly the resident bacteria of mucosal membranes (35, 36, 37). Collagen, as the most abundant protein in mammalians, is one of the main adhesion targets for a large number of bacteria (38). *T. pyogenes* also has the ability to attach to collagen through expression of collagen-binding protein (*cbpA*). Mutant strains of *Trueperella* which are unable to express *cbpA* show reduced adhesion to HeLa and 3T6 cells, whereas normal strains of *T. pyogenes* display higher adhesion qualities. Therefore, *cbpA* is considered as an important pathogenic factor required for adhesion and colonization of *T. pyogenes* to collagen-rich tissues (38). Fimbriae were determined in *T. pyogenes* for the first time by Jost and Billington in 2005 (2). Fimbriae of *T. pyogenes*, like other bacteria, promote the adhesion of this microorganism to the host tissues (9). In a study performed by Silva and others in 2008, 8 virulence factor-encoding genes were detected in *T. pyogenes* isolates including 4 fimbrial subtypes. In that study, *fimA, fimE* and both *fimC* and *fimG* were encoded, respectively, in 100%, 98% and 67% of isolates (16). In fact, the abundance of genes encoding fimbrial subtypes including *fimA, fimC, fimG* and *fimE* varies among different strains of *T. pyogenes*. Although, these virulence genes are encoded in both clinical and commensal *T. pyogenes* isolates, the difference is in the frequency of these genes in pathogenic conditions (3). For instance, in a study carried out by Santos in 2010 and ashrafi Tamai in 2017, a

higher frequency of *T. pyogenes fimA* gene was recorded in strains isolated from metritic cows in comparison to normal puerperium cows, indicating the importance of this gene in the adhesion of *T. pyogenes* and its pathogenesis (10, 11). In another study done by Bradely and others in 2015, all *T. pyogenes* isolated from cranial abscesses of male white-tailed deer encoded *fimA, plo* and *nanP*. However, *fimE, fimG, fimC* and *nanH* were only detected in 70% of those isolates and *cbp* had the lowest rate of expression (38). Magdalena and others in 2012 detected *plo* and *fimA* in all *T. pyogenes* isolated from *Bison bonasus* and they found that *fimG*, *fimC*, *cbp*, *nanP* and *nanH* were respectively encoded in 24%, 88%, 12%, 44% and 40% of the isolates (39). Ashraffi Tamai et.al. in 2017 detected *plo* and *fimA,* in all *T. pyogenes* isolated from cattle with clinical metritis and they found that *nanH*, *nanP*, *fimG*, *fimC*, *fimE* and *cbp* were respectively encoded in 83.1%, 76.9%, 61.5%, 69.2% , 76.9% and 56.9% of the isolates (11). Another aim of the present study was to determine the association between *T. pyogenes* strains isolated from different tissues and the aborted fetus. According to the result of Box PCR assay, all of the *T. pyogenes* isolates were clustered into one group while the control strain was in another cluster suggesting that all of the isolates were the same strain. Considering similar characteristics and virulence genes profiles of the isolates, it can be assumed that *Trueperella pyogenes* was the major causing pathogen in the described case.

In the current study, we discussed that although *T. pyogenes* is considered as a normal floral bacterium in various organs of domestic animals and the presence of virulence factors is not enough for its pathogenicity, it can turn into a primary pathogen depending on the host status and environmental conditions, and consequently, cause several complications including metritis, abortion and finally death in water buffalo (*Bubalus bubalis*). The genome sequence of this strain has been deposited in the GenBank database under the accession number CP028833.

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References

1. Yassin AF, Hupfer H, Siering C, Schumann P. Comparative chemotaxonomic and phylogenetic studies on the genus Arcanobacterium Collins et al. 1982 emend. Lehnen et al. 2006: proposal for Trueperella gen. nov. and emended description of the genus Arcanobacterium. Int J Syst Evol Microbiol 2011; 61: 1265–74.

2. Jost BH, Billington SJ. *Arcanobacterium pyogenes*: molecular pathogenesis of an animal opportunist. Antonie Van Leeuwenhoek 2005; 88: 87–102.

3. Rzewuska M, Kwiecień E, Chrobak-Chmiel D, Kizerwetter-Świda M, Stefańska I, Gieryńska M. Pathogenicity and virulence of *Trueperella pyogenes*: a review. Int J Mol Sci 2019; 20(11): e2737. doi: 10.3390/ijms20112737

4. Rogovskyy AS, Lawhon S, Kuczmanski K, et al. Phenotypic and genotypic characteristics of *Trueperella pyogenes* isolated from ruminants. J Vet Diagn Invest 2018; 30: 348–53.

5. Bicalho ML, Machado VS, Oikonomou G, Gilbert RO, Bicalho RC. Association between virulence factors of *Escherichia coli, Fusobacterium necrophorum* and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. Vet Microbiol 2012; 157: 125–31.

6. Moore R, Miyoshi A, Pacheco LGC, Seyffert N, Azevedo V. Corynebacterium and Arcanobacterium. In: Gyles CL, Prescott JF, Songer JG, Theone CO, eds. Pathogenesis of bacterial infections in animals. 4th ed. Ames : Wiley Blackwell, 2010: 113–47.

7. Gahrn HB, Frederiksen W. Human infections with *Actinomyces pyogenes* (*Corynebacterium pyogenes*). Diagn Microbiol Infect Dis 1992; 15: 349–54.

8. Plamondon M, Martinez G, Raynal L, Touchette M, Valiquette L. A fatal case of *Arcanobacterium pyogenes* endocarditis in a man with no identified animal contact: case report and review of the literature. Eur J Clin Microbiol Infect Dis 2007; 26: 663–6.

9. Liu M, Wang B, Liang H, Ma B, Wang J, Zhang W. Determination of the expression of three fimbrial subunit proteins in cultured *Trueperella pyogenes*. Acta Vet Scand 2018; 60: e53. doi: 10.1186/s13028-018-0407-3

10. Santos TMA, Caixeta LS, Machado VS, Rauf AK, Gilbert RO, Bicalho RC. Antimicrobial resistance and presence of virulence factor genes in *Arcanobacterium pyogenes* isolated from the uterus of postpartum dairy cows. Vet Microbiol 2010; 145: 84–9.

11. AshrafiTamai I, Mohammadzadeh A, ZahraeiSalehi T, Mahmoodi P. Genomic characterisation, detection of genes encoding virulence factors and evaluation of antibiotic resistance of *Trueperella pyogenes* isolated from cattle with clinical metritis. Antonie Van Leeuwenhoek 2018; 111: 2441–53.

12. Feßler AT, Schwarz S. Antimicrobial resistance in *Corynebacterium spp.*, *Arcanobacterium spp.*, and *Trueperella pyogenes*. Microbiol Spectr 2017; 5(6). doi: 10.1128/microbiolspec.ARBA-0021-2017

13. Al-Saggaf MS, Al-Ssum RM, Shair OMS. Isolation and identification of *Arcanobacterium pyogenes* (*Actinomyces pyogenes*) from Arabian gazelles. Afr J Biotechnol 2011; 10: 18614–31.

14. NCCLS Performance standards for antimicrobial susceptibility testing. 11th Informational Supplement, CLSI/NCCLS document M100-S11. Wayne, Pensylvania : National Committee for Clinical Laboratory Standards, 2001.

15. Ulbegi Mohyla H, Hijazin M, Alber J, et al. Identification of *Arcanobacterium pyogenes* isolated by post mortem examinations of a bearded dragon and a gecko by phenotypic and genotypic properties. J Vet Sci 2010; 11: 265–7.

16. Silva E, Gaivao M, Leitao S, et al. Genomic characterization of *Arcanobacterium pyogenes* isolates recovered from the uterus of dairy cows with normal puerperium or clinical metritis. Vet Microbiol 2008; 132: 111–8.

17. Martin B, Humbert O, Camara M, et al. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucl Acid Res 1992; 20: 3479–83.

18. Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl Environ Microbiol 1994; 60: 2286–95.

19. Quinn PJ, Markey BK, Leonard FC, Fitzpatrick ES, Fanning S, Hartigan PJ. Veterinary microbiology and microbial disease. 2nd ed. Oxford : Wiley Blackwell, 2011: 245–57.

20. Ribeiro MG, Risseti RM, Bolaños CAD, et al. *Trueperella pyogenes* multispecies infections in domestic animals: a retrospective study of 144 cases (2002 to 2012). Vet Q 2015; 35: 82–7.

21. Kasimanickam VR, Owen K, Kasimanickam RK. Detection of genes encoding multidrug resistance and biofilm virulence factor in uterine pathogenic bacteria in postpartum dairy cows. Theriogenology 2015; 30: 1–7.

22. Jensen R, Flint JC, Griner LA. Experimental hepatic necrobacillosis in beef cattle. J Am Vet Res 1954; 15: 5–14.

23. Nagaraja TG, Laudert SB, Parrott JC. Liver abscesses in feedlot cattle. Part I: causes, pathogenesis, pathology, and diagnosis. Compend Contin Educ Pract Vet 1996; 18: 230–41.

24. Nagaraja TG, Laudert SB, Parrott JC. Liver abscesses in feedlot cattle. Part IL Incidence, economic importance, and prevention. Compend Contin Educ Pract Vet 1996; 18: 264–73.

25. Narayanan S, Nagaraja TG, Staats J, Chengappa MM, Oberst RD. Biochemical and biological characterizations and ribotyping of *Actinomyces pyogenes* and *Actinomyces pyogenes*-like organisms from liver abscesses in cattle. Vet Microbiol 1998; 61: 289–303.

26. Narayanan S, Nagaraja TG, Wallace N, Staats J, Chengappa MM, Oberst RD. Biochemical and ribotypic comparison of *Actinomyces pyogenes* and *A. pyogenes*-like organisms from liver abscesses, ruminal wall, andruminal contents of cattle. J Am Vet Res 1998; 59: 271–6.

27. Hijazin M, Ulbegi-Mohyla H, Alber J, et al. Molecular identification and further characterization of *Arcanobacterium pyogenes* isolated from bovine mastitis and from various other origins. J Dairy Sci 2011; 94: 1813–9.

28. Rzewuska M, Stefańska I, Osińska B, et al. Phenotypic characteristics and virulence genotypes of *Trueperella* (*Arcanobacterium*) *pyogenes* strains isolated from European bison (*Bison bonasus*). Vet Microbiol 2012; 160: 69–76.

29. Zastempowska E, Lassa H, Genotypic characterization and evaluation of an antibiotic resistance of *Trueperella pyogenas* (*Arcanobacterium pyogenes)* isolated from milk of dairy cows with clinical mastitis. Vet Microbiol 2012; 161: 153–8.

30. Funke G, Ramos CP, Fernández-Garayzábal JF, Weiss N, Collins MD. Description of human-derived centers for disease control Coryneform group 2 bacteria as *Actinomyces bernardiae spp*. Int J Syst Bacteriol 1995; 45: 57–60.

31. Ding H, Lämmler C. Purification and fur-

ther characterization of a haemolysin of *Actinomyces pyogenes*. J Vet Med 1996; 43: 179–88.

32. Billington SJ, Jost BH, Cuevas WA, Bright KR, Songer JG. The *Arcanobacterium* (*Actinomyces*) *pyogenes* hemolysin, pyolysin, is a novel member of the thiolactivated cytolysin family. J Bacteriol 1997; 179: 6100–6.

33. Jost BH, Songer JG, Billington SJ. An *Arcanobacterium* (*Actinomyces*) *pyogenes* mutant deficient in production of the poreforming cytolysin pyolysin has reduced virulence. Infect Immun 1999; 67: 1723–8.

34. Gottschalk A, Bhargava AS. Neuraminidases. In: Boyer PD, ed. The enzymes. 3ed, Vol. 5. New York : Academic Press, 1971: 321–42.

35. Galen JE, Ketley JM, Fasano A, Richardson SH, Wasserman SS, Kaper JB. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. Infect Immun 1992, 60**:** 406–15.

36. Giebink GS. Otitis media: the chinchilla model. Microbial Drug Resist 1999; 5: 57–72.

37. Tong HH, Blue LE, James MA, DeMaria TF. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun 2000; 68: 921–4.

38. Esmay PA, Billington SJ, Link MA, Songer JG, Jost BH. The *Arcanobacterium pyogenes* collagen-binding protein, *CbpA*, promotes adhesion to host cells. Infect Immun 2003; 71: 4368–74.

39. Bradley SC, Emily HB, Shamus Pk, Michael JY, Karl VM. Isolation and genotypic characterization of *Trueperella (Arcanobacterium) Pyogenes* recovered from active cranial abscess infections of male white tailed deer (*Odocoileus Virginianus*). J Zoo Wildl Med 2015; 46: 62–7.

DOLOČITEV GENOTIPA SEVA *Trueperella pyogenes* KOT GLAVNEGA POVZROČITELJA VNETJA MATERNICE, SPLAVOV IN SMRTI PRI VODNIH BIVOLIH (*Bubalus bubalis*)

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Povzetek: *Trueperella pyogenes* je vrsta komenzalnih bakterij, ki je prisotna v sluznicah zgornjih dihal, urogenitalnega trakta in sluznicah prebavil pri govedu. Ta vrsta lahko samostojno ali v povezavi z drugimi vnetnimi bakterijami povzroči vnetja in ogroža zdravje. Sistemska oblika bolezni s splavom in smrtjo pri vodnih bivolih še ni bila dokumentirana. Pri samici vodnega bivola (*Bubalus bubalis*) z vnetjem maternice in pljučnico smo izolirali sev *T. pyogenes*, ki je povzročil splav in smrt prizadete živali. Raziskali smo virulenčne gene in njihovo odpornost proti antibiotikom. Z metodo PCR smo potrdili prisotnost dobro znanih virulenčnih genov *T. pyogenes*, vključno s plo, *nanH*, *nanP*, *cbpA*, *fimA*, *fimC*, *fimE* in *fimG* geni. Preizkus občutljivosti na antibiotike je pokazal, da je bil ta izolat odporen proti tetraciklinu, eritromicinu in trimetoprim sulfametoksazolu. Poleg tega smo z metodo Box-PCR ugotovili, da se vzorec DNK tega izolata razlikuje od vzorca kontrolnega seva (*T. pyogenes* ATCC 19411). Rezultati študije so pokazali, da lahko *T. pyogenes* povzroči sistemsko smrtonosno bolezen pri vodnih bivolih, vendar gostiteljski in okoljski pogoji pomebno vplivajo na razvoj okužbe. Kolikor vemo, je to prvo poročilo o bivolih s pljučnico, vnetjem maternice, splavom in smrtjo, ki jih je povzročil *T. pyogenes*.

Ključne besede: *Trueperella pyogenes; Bubalus bubalis;* bivol; BOX PCR; splav; smrt

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Original Research Article

EGG WEIGHT AFFECTS HATCHING RESULTS, BODY WEIGHT AND FEAR-RELATED BEHAVIOR IN JAPANESE QUAILS

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Abstract: This study was conducted to determine effect of egg weight on hatching results, chick performance and tonic immobility duration in Japanese Quails. Eggs were weighed individually and divided by weight into four groups: group 1 = 11.0 - 11.9 g, group 2 = 12.0 - 12.9 g, group 3 = 13.0 - 13.9 g and group 4 = 14.0 - 14.9 g. Egg weight loss during incubation and hatching parameters were recorded. Additionally, hatching weight and body weight were measured until 5 weeks and tonic immobility (TI) test was performed at the end of the experiment. Results revealed that egg weight loss during incubation decreased as egg weight increased. Group 2 was determined as the best suitable egg weight group in terms of fertility and group 2 and 3 in terms of hatchability of incubated eggs and hatchability of fertile eggs. Body weight increased as the egg weight increased and the heaviest chicks were hatched from heaviest egg group. Significant differences in TI duration between birds were noticed, as higher body weight birds had longer TI duration than birds with lower body weight. Thus, birds of high body weight were more fearful than birds of low body weight. It was concluded that the medium to heavy weight eggs are better eggs for hatching results and chick weight. Therefore, we recommend the selection of medium and heavy weight eggs for hatching implementations in Japanese quail.

Key words: body weight; egg weight; fertility; hatchability; tonic immobility

Introduction

Poultry production is the main source of household nutrition and income in the developing countries. Quail meat and eggs are considered a good source of animal protein that is very low in fat and cholesterol as compared to eggs of all other poultry species, which makes it the choice of people who suffer from high blood pressure (1). Egg weight is the most important factor affecting hatch weight in Japanese quail (2) and this is due to the positive correlation between the two traits (3, 4). Additionally, there are strong positive correlations, between poultry egg weights and hatching results (hatchability, embryonic mortality), hatching weights and subsequent performance of chicks (5-8). Egg weight also affects growth performance including the slaughter weight (9, 10). Thus, egg size is an important factor affecting homogeneity and uniformity of the flock.

Egg weight in Japanese quail ranges from 8.31 to 13.00g, as reported by Havenstein et al. (11). Hatching weight of Japanese quail chicks' increases with increased in egg weight. For example, the hatching weight of chicks originating from egg weight groups of 11.0 - 11.9, 12.0 -12.9 and 13.0 -13.9 g were as 6.98, 7.56 and 8.39 g, respectively (12). Other parameters impacted by egg weight include egg weight loss on 18th day

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of incubation, hatchability, and 7 days body weight in broilers (13). Seker et al (14) showed that highest levels of fertility and hatchability of incubated egg and hatchability of fertile egg were found in egg weight group of 10.5-11.5 g, followed by 11.51-12.50 g and then 9.50-10.50 g group. Furthermore, they found that increased egg weight was associated with significant increases in chick weight. Another study demonstrated that the least egg weight loss occurred in large egg size groups at different incubation periods with better fertility and hatchability traits recorded for medium egg weight and the heaviest chick weight was attained from large egg size of broiler breeder hens (15).

Since increases in egg weight positively correlate with higher chick weights, it has direct impact in enhancing all productive performances of chicks. Although different egg weight results in birds with different body weights, the criteria of how high or low body weight quails respond to different stressors is not well characterized. So we tried to investigate the relationship between body weight and fear related behavior of Japanese quails. Fear is widely regarded as a potent stressor that causes damaging effects on a number of performance indicators in poultry. There is mounting evidence for a positive association between fearfulness and adrenocortical activation in birds (16). Fearfulness is multidimensional trait that can be defined as a psychological profile resulting in an individual's consistent reactivity to fear-eliciting situation (17). Indeed, there are many studies showed that fear and fearfulness are associated with some performance indicators such as body weight and growth. Extreme or inappropriate expression of fear-related-behavior has negative effects on productivity and welfare in the domestic fowl (18). Reduction of fearfulness levels improves the bird's economic performance and ability for adaptation to environmental changes (19).

There are many tests to measure fearfulness level for birds. The test used in present study is tonic immobility test (TI). Many invertebrate and vertebrate animals, upon release from brief physical restraint, do not attempt to escape but instead, remain in an immobile state characterized by relative muscular hyper tonicity, intermittent eye closure, and depressed heart rate (20). This phenomenon has been labeled variously as death feigning, animal hypnosis and more recently TI. Fear can be assessed by measuring the duration of TI reaction (21). TI is a variable period of immobility induced by manual restraint. A long duration of TI is indicative of high levels of fearfulness, and a short duration is indicative of low levels of fearfulness (22).

In Japanese quail, fear-related behavior studied for lines divergently long term selected for high or low body weight at four weeks of age. They found that low body weight quail line showed greater avoidance of conspicuous novel objects placed near the home cage and longer tonic immobility fear reactions, which included vocalizing and struggling later and less often during mechanical restraint than did the control quail and those with high body weight (23). Generally, low body weight quails showed intermediate responses and greater plasma corticosterone concentrations following mechanical restraint when compared to control non selected line or high body weight groups. Mills et al. (24) demonstrated that lines of Japanese quail which have been divergently selected for plasma corticosterone levels in response to restraint reared in crush cage showed marked differences in their fear responses. Moreover, birds' lines selected for high response to restraint in relation to plasma corticosterone levels show longer duration of the tonic immobility reaction than did lines selected for low response to restraint.

Therefore, the objectives of the present study were to determine 1) the effects of egg weight on egg weight loss during incubation, hatching results and subsequent Japanese quail performance (body weight), and 2) the relationship between body weight and fear related behavior as measured by the duration of TI reaction.

Materials and methods

The experiment was carried out at department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary medicine, Alexandria University, Egypt. A total of 1200 fertile eggs of (non-genetically selected) Japanese quails (*Coturnix coturnix japonica*) were purchased from a private farm. Eggs were weighed individually and divided by weight into four groups (300 eggs per group): group 1= 11.0 - 11.9 g, group 2 = 12.0 - 12.9 g, group 3 = 13.0 - 13.9 g and group 4= 14.0 - 14.9 g. Eggs were fumigated and incubated vertically with broad end up in the setting

trays in standard incubator at 37.5˚C (dry bulb temperature), with 65% relative humidity. Eggs were turned automatically with turning angle ± 45 degree from vertical position eight times daily using automatic timer. On day 15 of incubation, the eggs were transferred to the hatchery at 36.5˚C (dry bulb temperature), with 75-80% relative humidity. At the time of hatch (18th day of incubation), chicks were wing banded from each group and randomly distributed in rearing rooms. The chicks were brooded at a temperature of 35˚C using automatic gas heaters as a source of heat. Temperature was gradually reduced weekly by 3˚C until room temperature reached 24˚C at the fifth week. Chicks were fed *ad libitum* commercial starter diets containing 22% crude protein and 3100 kcal of metabolizable energy meeting requirements (25). Chicks were allowed free access to water as well. Lighting regime was 24 hours (2.5 foot candle) from first day until day seven, and then reduced to 8 hours (0.5 foot candle) until end of the experiment.

Parameters:

Egg Weight Loss during Incubation

Eggs (60 eggs/ group) were weighed just prior to setting into incubator. At 5, 10 and 15 days of incubation eggs were removed from incubator and weighed using an electronic scale to determine average egg weight and egg weight loss percentages. Weight loss (%) = [(egg weight at setting – egg weight at different days of incubation)/ egg weight at setting].

Hatching Parameters

At the end of the incubation period, the eggs having no chick release (Unhatched) were cracked to determine the fertility, and hatchability of incubated eggs and the hatchability of fertile eggs. Calculations were made as follows:

Fertility (%) = (number of fertilized eggs / total numbers eggs placed into incubator) x 100.

Hatchability of incubated eggs (%) = (number of released chicks / total number of egg placed into incubator) x 100.

Hatchability of fertile eggs (%) = (number of released chicks / number of fertilized eggs placed into incubator) x 100.

Body Weight

A total number of 600 chicks were used with 150 bird/ group. Hatched chicks were wing-banded and weighed at hatch. Body weight was recorded every week until 5th week. At age of three weeks, the sex of each bird was determined and recorded.

Tonic Immobility Test (TI):

A sample of 16 random mixed sex birds with sex ratio 1:1 (8 males and 8 females) from each group with total number 64 birds was induced into tonic immobility at 5 week of age. Each bird was tested individually and once in a separate room. TI was induced by inverting the birds and restraining it for 15 s on a table on their back, raising their necks by a towel warped underneath; their head was dropped down from the edge of the table. One hand rested on the sternum while the other lightly cupped the head. The time elapsed till the bird raised up again and number of inductions did by the bird for rising was recorded using stop watch to record latencies until the bird righted itself. If TI could not be induced after 5 consecutive attempts, the bird was considered to be non-susceptible. If the bird failed to right itself after 10 minutes, a maximum score of 600 s was given.

Statistical Analysis:

Data for egg weight loss, fertility and hatchability were analyzed using one way ANOVA while, data on body weight, tonic immobility test were analyzed by Two-way ANOVA. In case of significant differences ($P<0.05$), means were compared by Duncan's test using SAS statistical package (26). Statistical model for two way ANOVA: $X_{ijk}=\mu + A_{i} + B_{j} + (AB)_{ij} + e_{ijk}$

Where: X_{ijk} = Value of ith observation (body weight, tonic immobility test) of the ith group; μ = Overall mean; Ai = Effect of ith group (different egg weight group); B_j = Effect of jth sex of birds; (AB) ij = Effect of interaction between ith egg weight and the jth sex of birds; e_{ijk} = random error.

Table 1: Effect of Japanese quail egg weight on egg weight loss during incubation period

Group of Eggs weight	Egg weight at time of incubation (g)	Egg weight loss (%) at different incubation periods *			
		0-5 days	5-10 days	10-15 days	0-15 days
G1 (11.0-11.9g)	11.50± 0.30^{d}	2.97±0.73^{ab}	3.61±1.23^{a}	2.80±1.10	9.37±1.73^{a}
G2 (12.0-12.9 g)	12.46±0.24^{c}	3.35±1.40^{a}	2.74±0.81^{b}	3.10±1.24	9.19±2.16^{a}
G3 (13.0-13.9 g)	13.39±0.34^{b}	3.14±1.05^{a}	2.55±1.21^{b}	2.94±1.16	8.63±1.92^{ab}
G4 (14.0-14.9 g)	14.39±0.36^{a}	2.60±0.87^{b}	2.74±1.25^{b}	2.86±1.08	8.20±2.50^{b}
P value	<.0001	0.0068	<.0001	0.6273	0.0301

Means bearing different letters within the same column are significantly different (P<0.05). Data were shown as mean ±SD.*Egg weight loss (%) = [(egg weight at setting – egg weight at different days of incubation)/ egg weight at setting

Table 2: Effect of Japanese quail egg weight on hatching result

Group of Eggs Weight	Fertility (%)[1]	Hatchability of incubated eggs (%)[2]	Hatchability of fertile eggs (%)[3]
G1 (11.0-11.9 g)	87.22 ± 4.24^{c}	63.76 ± 14.77^{b}	71.91 ± 12.13
G2 (12.0-12.9 g)	96.11 ± 1.78^{a}	77.77 ± 7.87^{a}	80.89 ± 8.81
G3 (13.0-13.9 g)	91.91 ± 1.67^{b}	74.76 ± 4.75^{a}	80.60 ± 6.19
G4 (14.0-14.9 g)	93.00 ± 2.47^{b}	71.72 ± 6.96^{ab}	76.81 ± 7.49
P value	<.0001	0.0212	0.1363

Means bearing different letters within the same column are significantly different (P<0.05). Data were shown as mean ±SD. [1]Number of fertile eggs/ number of eggs set × 100. [2]Number of hatched chicks /number of total eggs set × 100. [3]Number of hatched chicks /number of fertile eggs set × 100

Table 3: Means ±SD of Japanese quail chick's body weight as affected by eggs weight and sex of the bird

Group of Eggs Weight	Sex	Weight (g)/ age					
		Hatch Weight	Week 1	Week 2	Week 3	Week 4	Week 5
G1 (11.0-11.9 g)	F	8.70±0.54^{a}	19.12±2.38	39.59±4.70	92.60±7.24^{a}	140.45±17.45^{a}	183.64±18.94^{a}
	M	8.53±0.37^{b}	18.57±2.59	39.44±4.75	80.19±7.66^{b}	131.23±10.46^{b}	161.23±18.46^{b}
Overall		8.58 ±0.43^{D}	18.73 ± 0.53^{D}	39.48 ± 4.72^{D}	83.83 ± 9.41^{D}	133.93 ±13.52^{C}	167.80 ± 21.18^{C}
G2 (12.0-12.9 g)	F	9.24±0.39	20.25±2.50	43.06±4.81	88.81±11.53	136.97±15.65	178.88±19.20^{a}
	M	9.25±0.30	20.00±2.46	41.94±6.02	85.60±10.94	140.72±10.65	173.31±11.54^{b}
Overall		9.24 ±0.34^{C}	20.11 ± 2.47^{C}	42.44 ± 5.52^{C}	87.04 ±11.28^{C}	139.05 ± 13.21^{B}	175.33 ± 16.40^{B}
G3 (13.0-13.9 g)	F	10.01±0.46	21.43±3.01	44.03±7.10	89.82±14.04	138.62±17.97	178.59±22.62
	M	10.00±0.45	21.00±2.45	44.75±6.49	92.33±11.09	140.22±15.00	178.36±17.43
Overall		10.01 0.45^{B}	21.27 ± 2.81^{B}	44.31 ± 6.86^{B}	90.79 ±13.00^{B}	139.24 ± 16.85^{B}	179.33 ± 33.60^{B}
G4 (14.0-14.9 g)	F	10.62±0.46^{a}	23.85±2.69^{a}	48.81±5.73^{a}	95.90±12.42^{a}	150.25±18.72^{a}	191.78±18.56^{a}
	M	10.40±0.71^{b}	22.85±2.14^{b}	46.60±4.59^{b}	88.90±12.38^{b}	137.72±18.79^{b}	177.07±13.52^{b}
Overall		10.55 0.55^{A}	23.55 ± 2.57^{A}	48.13 ± 5.48^{A}	93.75 ± 2.78^{A}	146.41 ± 19.56^{A}	186.07 ± 20.82^{A}

Means bearing different letters within the same column are significantly different (P<0.05). Data were shown as mean ±SD

Table 4: Effect of eggs weight and sex of the bird on Tonic immobility test (TI) reaction of Japanese quail

Group of Eggs Weight	Sex	No. of inductions	Duration till self-righting (s)
G1 (11.0-11.9 g)	F	1.10+0.15	31.57±1.29[a]
	M	1.11 ± 0.17	26.17 ± 2.07 [b]
Overall		1.11 ± 0.10	33.58 ± 2.84[C]
G2 (12.0-12.9 g)	F	1.15+0.13	48.52±3.97[a]
	M	1.17 ± 0.01	44.37±2.37 [b]
Overall		1.18 ± 0.13	47.07 ± 3.28[B]
G3 (13.0-13.9 g)	F	1.20± 0.19	53.45±4.55[a]
	M	1.24± 0.04	46.02±2.54[b]
Overall		1.24 ± 0.11	51.95 ± 4.21[B]
G4 (14.0-14.9 g)	F	1.27± 0.06	65.54±3.93[a]
	M	1.32± 0.18	57.34±4.82[b]
Overall		1.32 ± 0.18	67.25 ± 6.52[A]

Means bearing different letters within the same column are significantly different (P<0.05). Data were shown as mean ±SD. No = number, s = seconds

Results

Data on egg weight losses during incubation period among different egg weight groups of Japanese quail are presented in Table 1. Significant differences were found during first five days of incubation (P = 0.0068) and from 5th to 10th day of incubation (P<.0001) in egg weight loss of different egg weight groups. The weight loss from 10th to 15th day of incubation was not significant (P = 0.6273). However, the overall percentage of egg weight loss for entire period of incubation was significantly (P = 0.0301) different among egg weight groups.

Data on effects of egg weight on hatching results are presented in Table 2. The effect of egg weight on fertility (P < 0.0001) and hatchability of incubated eggs (P = 0.0212) were significant. However, the hatchability of fertile egg (P = 0.1363) was not significant.

Average body weight within different egg weight groups are presented in Table 3. The effects of egg weight on quail hatching weight, one week, two weeks, three weeks, four weeks, and five weeks body weights were significant (P<.0001). However, the effect of sex on body weight for group 1 was significant at hatch weight, 3rd, 4th and 5th week of age with females being heavier than males. In Group 2, there was no difference in body weight between males and females. In group 3, significant difference in body weight between male and females was observed at age of 5th week only. In group 4, significant difference between males and females was observed from hatch till 5th week with females being heavier than males.

In Table 4, results of effect of egg weight on (TI) are presented. Birds with higher body weight (Group 4) showed more fearfulness than birds with lower body weight in (Group 1) as it need longer TI duration for bird's up-righting. With increased bird weight, TI duration increased as in Groups 2 and 3 (vs Group 1). While, there was no significant difference in number of inductions required to straight between groups. Moreover, sex significantly affects TI duration as it was higher in females than in males in all groups.

Discussion

Percentage of weight loss decreased as egg weight increased. The lowest percentage of weight loss in large eggs can be attributed to the greater percentage of albumen content in large eggs. Egg weight loss that occurs during incubation of eggs is due to water diffusion through the shell (27). Our observation in Japanese quail is similar to observations in Cobb 500 broiler breeder hen (28). Deeming (29) determined that 10–12% weight loss is necessary during incubation in order to get a good incubation result in stored and non-stored eggs.

In the current study, the higher percentage of egg weight loss during the entire period of incubation was recorded for group 1 and 2 (9.37% and 9.19%, respectively, followed by 8.63 and 8.20

% in group 3 and 4 that is lower than the value of 12.94% for quail eggs (30). It is also lower than the values of 24.76% and 20.90% in quail eggs (31, 32). Egg weight loss is an important parameter for incubation and it has been used to estimate vital gas exchange (33). It is also correlated with embryo metabolism and development rates (34). Gonzalez et al. (35) noted that increased egg weight loss is positively correlated with increased egg weight. They attributed the increased loss to surface area of the egg and high demand for energy needed for the embryonic development. It was reported that for every gram of fat burned from the stored fat in the yolk, an almost equal mass of metabolic water is generated.

The higher fertility percentage in this study was observed in group 2 (96.11%) followed by group 4 (93%), group 3 (91.91%) and group 1 (87.22%). The same trend was noticed in hatchability, where the maximum hatchability was recorded in group 2 and 3 followed by group 4 and the lower percentage was recorded in group 1. This finding is similar to that of Rashid et al. (36) who reported that percentage hatchability of medium-sized eggs was higher than those in large sized eggs in chickens. Similarly, Iqbal et al. (15) reported that better fertility and hatching traits were attained in medium egg size in broiler breeder.

In the present study, maximum fertility and hatchability of Japanese quail egg were observed in group 2 (12.0-12.9 g) and heavier group while the minimum values were recorded in group 1 (11.0-11.9 g). This was in agreement with Taskin et al. (37) reported that fertility percentage was affected by quail egg weight categories; the highest value (91%) was recorded for heaviest group (>13.00g). Another study showed that the highest fertility and hatchability of incubated eggs of Japanese quails was observed with the eggs in the weight of 11.6g and greater (38). They also found the highest hatchability of fertile eggs with lighter eggs in the weight of 10.6-11.5g. They determined the lowest level of fertility, hatching and hatchability of fertile eggs in eggs that were in the weight of 9.5g and lower.

With regard to body weight of Japanese quail chicks, significantly higher body weight was recorded for the heavier egg group and this trend continued from hatch weight till 5th week body weight. Our findings are in agreement with Dudusola (39) who reported that the chick weight of Japanese quail increased significantly as a result of the increasing egg size. Similarly, Abiola et al. (40) reported that small chicks were hatched from small eggs while large chicks were hatched from large eggs in broiler breeder. The improved growth performance of chicks from heavier eggs may be attributed to the mass of the residual yolk sac that the chick retains at hatching (41, 42, 43 and 44).

Regarding the effect of sex, our data showed that there was a significant difference between males and females in three groups (1,3 and 4) at 5th week of age. Similar findings documented that females of Japanese quails were heavier than males (2 ,45, 46 and 47). The differences in body weight between males and females may attributed to the fact that females grow faster and yield larger muscles and more abdominal fat than males at the same age (48). Subtle differences in the biology of the two sexes, such as the functions of hormonal and regulatory systems may also differentially impact the growth and development of the two sexes at early ages as it is the case in most species of animals (49).

Fear is considered a strong emotion that exerts a huge inhibition on behavioral patterns generated by all other motivational systems (50, 51). Generally, increasing fear is characterized by increased withdrawal, silence and inactivity (52). Longer tonic immobility reactions and more pronounced silence and inactivity are all due to elevated levels of fearfulness (53, 54 and 55).

In present study, higher body weight quails showed longer tonic immobility duration a trait that is associated with high level of fearfulness (56). This was manifested by longer time for self-righting than in birds with lower body weight. Recoquillay et al. (57) reported that the duration of tonic immobility was positively correlated with weight at 17 and 65 days of age (R = 0.76 and 0.79, respectively) in Japanese quail resulted from second generation crossing of two lines divergently selected for their social reinstatement behavior. In contrast, others observed that fearfulness is higher in quails with lower body weight bird than in higher body weight at 4th week of age (58, 59). Jones et al. (23) also found that low body weight quail lines showed greater avoidance of conspicuous novel objects and were more fearful than quails with high body weight. On the other hand, some studies reported that there is no relationship between body weight and TI duration as body mass did not significantly affect TI duration in Japanese quails (60).

The development of tonic immobility response varies between the sexes. TI duration was longer in females than in males indicating that female quails are more fearful than males. A similar finding was supported by Pittet et al. (61) who stated that female is more fearful than males in quails. Since females have higher body weight than males, this could be responsible for the prolonged TI duration. This trait could also be attributed to estrogen hormone in females while in males, the presence of more activity and reduced fearfulness may attributed to testosterone hormone as evidenced by reduced fearfulness of Japanese quail's chicks produced following injection of testosterone in to the yolk (62).

Conclusion

We found that Group 2 (12-12.9g) was as the most suitable egg weight group in terms of fertility and the group 2 and 3 (13-13.9g) in terms of hatchability of incubated eggs and hatchability of fertile eggs. The heaviest chicks were obtained from the heaviest eggs (weight of 14.0-14.9g). Moreover, birds with higher body weight had longer TI duration and were more fearful than birds with lower body weight. It is concluded that medium to heavy weight eggs are associated with better hatching outcomes and chick growth. Therefore, we recommend the selection of medium and heavy weight eggs for hatching implementations in Japanese quails.

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References

1. Rogerio GT. Quail meat-an undiscovered alternative. World Poult J 2009; 25(2): 7–16.

2. Saatci, M, Omed H, Dewi IA. Genetic parameters from univariate and bivariate analysis of egg and weight traits in japanese quail. Poult Sci 2006; 85: 185–90.

3. Kselen LVI, Pannia LVI. The correlation of egg weight with hatching weight and 56 days weight of broiler fowls. Anim Breed Abstr 1976; 44(2): 888.

4. Kucukyilmaz K, Baser E, Erensayin C, Orhan H, Arat E. Effect of egg weight on the hatchability, fattening performance and egg yield traits of Japanese quail. J Cent Anim Res Inst 2001; 11: 6–12.

5. Witt de F, Schwalbach LMJ. The effect of egg weight on the hatchability and growth performance of new hampshire and rhode ısland red chicks. S Afr J Anim Sci 2004; 34(2): 62–4.

6. Alkan S, Karabag K, Galic A, Balcioglu MS. Effects of genotype and egg weight on hatchability traits and hatching weight in Japanese quail. S Afr J Anim Sci 2008; 38(3): 231–7.

7. Çağlayan T, Garip M, Kırıkçı K, Günlü A. Effect of egg weight on chickweight, egg weight loss and hatchability in rock partridges (*A. graeca*). Ital J Anim Sci 2009; 8(4): 567–74.

8. Alabi OJ, Ng'ambi JW, Norris D, Mabelebele M. Effect of egg weight on hatchability and subsecuent performance of Potchefstroom koekoek chicks. Asian J Anim Vet Adv 2012; 7(8): 718–25.

9. Petek M, Baspinar H, Ogan M. Effects of egg weight and length of storage on hatchability and subsequent growth performance of quail. S Afr J Anim Sci 2003; 33 (4): 242–7.

10. Nazlıgül A, Türkyılmaz MK, Bardakçıoğlu HE. Effects of hatching egg weight on hatching chick weight, post hatching growth performance and livability in Japanese quails (*Coturnix coturnix japonica*). İstanbul Üniv Vet Fak Derg 2005; 31(2): 33–40.

11. Havenstein GB, Nestor KE, Bacon WL. Comparison of pedigreed and non- pedigreed random-bred control systems for use with artificial selection in Japanese quail. Poult Sci 1988; 67(3): 357–66.

12. Yildirim I, Yetisir R. Effects of hatching egg weight and parental age on the hatching weight and 6th week live weight in Japanese quail (*Coturnix coturnix japonica*). Turk J Vet Anim Sci 1998; 22: 315–9.

13. Duman M, Şekeroğlu A. Effect of egg weights on hatching results, broiler performance and some stress parameters. Braz J Poult Sci 2017; 19(2): 255–62.

14. Seker I, Kul S, Bayraktar M. Effects of parental age and hatching egg weight of Japanese quails on hatchability and chick weight. Int J Poult Sci 2004; 3: 259–65.

15. Iqbal J, Khan SH, Mukhtar N, Ahmed T, Pasha RA. Effects of egg size (weight) and age on hatching performance and chick quality of broiler breeder. J Appl Anim Res 2016; 44(1): 54–64.

16. Harvey S, Phillips JG, Res A, Hall TR. Stress and adrenal function. J Exp Zool 1984; 232: 633–45.

17. Boissy A. Fear and fearfulness in animals. Q Rev Biol 1995; 70: 165–91.

18. Jones RB, Hocking PM. Genetic selection for poultry behaviour: big bad wolf or friend in need? Anim Welf 1999; 8: 343–59.

19. Faure JM, Mills AD. Improving the adaptability of animals by selection. In: Grandin T, editor. Genetics and the behavior of domestic animals. San Diego : Academic Press, 1998: 235–64.

20. Gallup GG, Maser JD. Catatonia: tonic immobility: evolutionary underpinnings of human catalepsy and catatonia. In: Maser JD, Seligman MEP, eds. Psychopathology: experimental models. San Francisco : W. H. Freeman and Company, 1977: 334–57.

21. Gallup GG. Tonic immobility as a measure of fear in domestic fowl. Anim Behav 1979; 27: 316–317.

22. Jones RB. The tonic immobility reaction of the domestic fowl: a review. Worlds Poult Sci J 1986; 42: 82–96.

23. Jones RB, Satterlee DG, Marks HL. Fear-related behaviour in Japanese quail divergently selected for body weight. Appl Anim Behav Sci 1996; 52: 87–98.

24. Mills AD, Crawford LL, Domjan M, Faure JM. The behaviour of Japanese or domestic quail *Coturnix japonica*. Neurosci Biobehav Rev 1997; 21: 261–81.

25. NRC. Nutrient requirements of poultry. 9th revised ed. Washington : National Academy Press, 1994.

26. SAS Institute. Statistical user's guide' statistical analysis system. INT., Cary, NC. U.S.A., 2014.

27. Tona K, Bamelis F, Couke W, Bruggeman V, Decuypere E. Relationship between broiler breeders age and egg weight loss and embryonic mortality during incubation in large scale conditions. J Appl Poult Res 2001; 10: 221–7.

28. Ulmer-Franco AM, Fasenko GM, O'Dea Christopher EE. Hatching egg characteristics chick quality and broiler performance at 2 breeder flock ages and from 3 egg weights. Poult Sci 2010; 89: 2735—42.

29. Deeming DC. Factors affecting hatchability during commercial incubation of ostrich (*Struthio camelus*) eggs. Br Poult Sci 1995; 36: 51–65.

30. Soliman FNK, Rizk RE, Brake J. Relationship between shell porosity, shell thickness, egg weight loss, and embryonic development in Japanese quail eggs. Poult Sci 1994; 73: 1607–11.

31. Saylam SK. The effects of egg weight and storage time on egg weight loss and hatchability traits in Japanese quail. Turk J Vet Anim Sci 1999; 23: 367–72.

32. Saylam SK, Sarıca M. Effects of shell thickness, shell pores and egg weight loss on hatchability on Japanese quail eggs. Turk J Vet Anim Sci 1999; 23: 41–6.

33. Rahn H, Ar A, Paganelli CV. How bird eggs breathe. Sci Am 1979; 240: 46–55.

34. Burton FG, Tullet SG. A comparison of the effect of eggshell porosity on the respiration and growth of domestic fowl, duck and turkey embryos. Comp Biochem Physiol1983; 75(A): 167–74.

35. Gonzalez A,. Satterlee DG, Moharer F, Cadd GG. Factors affecting ostrich egg hatchability. Poult Sci 1999; 78:1257–62.

36. Rashid A, Khan SH, Abbas G, Amer MY, Khan MJ, Iftikhar N. Effect of egg weight on hatchability and hatchling weight in Fayoumi, Desi and crossbred (Rhode Island Red × Fayoumi) chickens. Vet World 2013; 6: 592–5.

37. Taskin A, Karadavut U, Cayan H, Genc S, Coskun I. Determination of small variation effects of egg weight and shape index on fertility and hatching rates in japanese quail (*Coturnix coturnix japonica*). J Selcuk Univ Nat Appl Sci 2015; 4(2): 73–83.

38. Sarica M, Soley F. The effect of hatching egg weight on the hatchability, growing and egg production traits of Japanese quail (*Coturnix coturnix japonica*). J Agric Fac O.M.U 1995; 10: 19–30.

39. Dudusola IO. The effect of parental age and egg weight on fertility, hatchability and day-old chick weight of Japanese quail (*Coturnix coturnix japonica*). Stand Res J Agric Sci 2013; 1(2): 13–6.

40. Abiola SS, Meshioye OO, Oyerinde BO, Bamgbose MA. Effect of egg size on hatchability of broiler chicks. Arch Zoot 2008; 57: 83–6.

41. Abiola SS. Effects of turning frequency of hen's egg in electric table-type incubator on weight losses, hatchability and mortality in Nigeria. Agric J 1999; 30: 77–82.

42. Donald D, Bell WD, Weaver JR. Commercial meat and egg production. 5th ed. Norwell (MA); Kluwer Academic Publishers, 2002: 641–3.

43. Rashid MM, Islam MN, Roy BC, Jakobsen K, Lauridsen C. Nutrient concentrations of crop and gizzard contents of indigenous scavenging chickens under rural conditions of Bangladesh. Livest Res Rural Dev 2005; 17(4): e16. http://www.lrrd.org/lrrd17/2/rash17016.htm (Dec. 2019)

44. King'ori AM, Tuitoek JK, Muiruri HK, Wa-

chira AM. Protein requirements of growing indigenous chickens during the 14–21 weeks growing period. S Afr Soc Anim Sci 2007; 33:78–81.

45. Alkan S, Karabag K, Galic A, Karsli T, Balcioglu MS. Determination of body weight and some carcass traits in japanese quails (*Coturnix coturnix japonica*) of different lines. Kafkas Univ Vet Fak Derg 2010; 16(2): 277–80.

46. Tarhyel R, Tanimomo BK, Hena SA. Effect of sex, color and weight group on carcass characteristics of Japanese quail. Sci J Anim Sci 2012; 1(1): 22–7.

47. Olawumi SO. Effects of housing and sex on growth performance of coturnix quails in the derived savannah zone of Nigeria. Int J Agric Forest Fish 2015; 3(6): 227–31.

48. Caron N, Minvielle F, Desmarais M, Poste LM. Mass selection for 45 day body weight in Japanese quail selection response, carcass composition, cooking properties and sensory characteristics. Poult Sci 1990; 29:1038–45

49. Vasilatos-Younken R, Bacon WL, Nestor KE. Relationship of plasma growth hormone to growth within and between turkey lines selected for different growth rates. Poult Sci 1988; 67: 826–34.

50. Hogan JA. An experimental study of conflict and fear: an analysis of behaviour of young chicks towards a mealworm: Part 1. The behaviour of chicks which do not eat the mealworm. Behaviour 1965; 26: 45–95.

51. Jones RB. Fear and adaptability in poultry: insights, implications and imperatives. Worlds Poult Sci J 1996; 52: 131–74.

52. Jones RB. The assessment of fear in the domestic fowl. In: Zayan R, Duncan IJH, eds. Cognitive aspects of social behaviour in the domestic fowl. Amsterdam : Elsevier, 1987: 740–81.

53. Hughes BO, Black AJ. The effect of environmental factors on activity, selected behaviour patterns and "fear" of fowls in cages and pens. Br Poult Sci 1974; 15: 375–80.

54. Craig JV, Craig TP, Dayton AD. Fearful behavior by hens of two genetic stocks. Appl Anim Ethol 1983; 10: 263–73.

55. Jones RB, Mills AD, Faure JM, Williams JB. Restraint, fear and distress in Japanese quail genetically selected for long or short tonic immobility reactions. Physiol Behav 1994; 56: 529–34.

56. Jones RB. The nature of handling immediately prior to test affects tonic immobility fear reactions in laying hens and broiler. Appl Anim Behav Sci 1992; 34: 247–54.

57. Recoquillay J, Leterrier C, Calandreau L, et al. Evidence of phenotypic and genetic relationships between sociality, emotional reactivity and production traits in Japanese quail. PLoS One 2013; 8(12): e82157. doi: 10.1371/journal.pone.0082157

58. Darden JR, Marks HL. Divergent selection for growth in Japanese quail under split and completes nutritional environments. 3. Influence of selection for growth on heterotic effects for body weight, feed and water intake patterns, abdominal fat and carcass lipid characteristics. Poult Sci 1989; 68: 37–45.

59. Mills AD, Faure JM. Panic and hysteria in domestic fowl: a review. In: Zayan R, Dantzer R, eds. Social stress in domestic animals. Dordrecht : Kluwer Academic Publishers, 1990: 248–72.

60. Calandreau L, Favreau-Peigne A, Bertin A, et al. Higher inherent fearfulness potentiates the effects of chronic stress in the Japanese quail. Behav Brain Res 2011; 255: 505–10.

61. Pittet F, Houdelier C, Lumineau S. Precocial bird mother shape sex differences in the behavior of chicks. J Exp Zool 2014; 312A: 265–75.

62. Daisley J, Bromundt V, Möstl E, Kotrschal K. Enhanced yolk testosterone influences behavioral phenotype independent of sex in Japanese quail chicks *Coturnix japonica*. Horm Behav 2005; 47: 185–94.

TEŽA JAJC VPLIVA NA NESNOST, TELESNO TEŽO TER VEDENJE POVEZANO S STRAHOM PRI JAPONSKIH PREPELICAH

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Povzetek: Študija je bila izvedena z namenom določanja vpliva teže jajc na rezultate izvalitve, uspešnost piščancev in trajanje tonične negibnosti pri japonskih prepelicah. Jajca smo posamezno stehtali in jih po teži razdelili v štiri skupine: skupina 1 = 11,0 - 11,9 g, skupina 2 = 12,0 - 12,9 g, skupina 3 = 13,0 - 13,9 g in skupina 4 = 14,0 - 14,9 g. V času valjenja so bile zabeležene izgube teže jajc ter parametri valjenja. Izmerili smo tudi težo valilne mase in telesno maso do 5 tednov starosti, na koncu poskusa pa izvedli test tonične negibnosti (TI). Rezultati so pokazali, da se je izguba teže jajca med inkubacijo zmanjševala s povečanjem teže jajc. Skupina 2 je bila kot najbolj primerna skupina jajc glede plodnosti, skupini 2 in 3 pa glede na valilnost inkubiranih jajc in valilnost plodnih jajc. Telesna teža se je povečevala, ko se je povečala teža jajc in najtežji piščanci so se izvalili iz najtežje skupine jajc. Opažene so bile pomembne razlike v trajanju TI med pticami, saj so imele ptice z višjo telesno težo daljše trajanje TI kot ptice z nižjo telesno težo. Tako je bilo ptice z veliko telesno težo bolj strah kot ptice z nizko telesno težo. Ugotovljeno je bilo, da so jajca srednje do višje teže boljša jajca glede valjenja in teže piščancev. Zato priporočamo izbiro jajc srednje in višje teže za valjenje japonskih prepelic.

Ključne besede: telesna teža; teža jajc; plodnost; valilnost; tonična negibnost

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Original Research Article

INVESTIGATION OF G+265C AND G-1539A SINGLE NUCLEOTIDE POLYMORPHISMS OF TOLL-LIKE RECEPTOR 4 GENE (TLR4) IN SOME CATTLE BREEDS RAISED IN TURKEY

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Abstract: Toll-like receptors play an essential role in how the innate immune system reacts to pathogens. These receptors help the innate immune system recognise the antigenic structure of pathogens and initiate the inflammatory response. TLR4 is one of these receptors, and it has been identified as a candidate molecular marker for resistance to mastitis in cattle. This study aimed to describe G-1539A (BglI) in promoter and G+265C (MspI) in 5'-UTR polymorphisms in the TLR4 gene using Zavot (n=60), East Anatolian Red (EAR, n=49), Anatolian Black (AB, n=59), South Anatolian Red (SAR, n=42), Turkish Gray (TG, n=60) and Holstein (n=218) cattle breeds. For this purpose, we used polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) to genotype a total of 488 cattle. Two alleles (A and G) and three genotypes (GG, GA and AA) were examined by digestion of the PCR product with BglI restriction enzyme. The frequency of the G allele was higher in all investigated breeds except Zavot. The GA genotype was found to be the most common genotype in the EAR, SAR, TG, AB, Holstein, and Zavot breeds. Two alleles (C and G) and three genotypes (GG, GC and CC) were examined by digestion of the PCR product for the G+265C SNP with the MspI restriction enzyme. The most prevalent in terms of frequency was the C allele in all breeds with regard to G+265C SNP. All examined breeds were within the Hardy-Weinberg equilibrium ($p>0.05$). Consequently, the existence of two SNPs of the TLR4 gene has been reported for the first time in six cattle breeds raised in Turkey. The study demonstrated that the investigated breeds continue to exhibit variation in terms of these two SNPs. It might be possible to utilise these polymorphisms in efforts to breed herds resistant to significant breeding diseases, such as mastitis.

Key words: cattle; PCR-RFLP; promoter; SNP; TLR4

Introduction

As an effect of antagonistic genetic correlations between milk yield and immunity, a decline in resistance to disease has been observed in highly productive herds (1, 2). The goal of high profitability has complicated the issue of selecting the most appropriate method for production as well as fertility management, udder health, and resistance to disease while maximising profit without compromising animal welfare. Recent studies have shown that creating herds resistant to serious breeding diseases, such as mastitis, tuberculosis, and brucellosis, could increase the profitability of livestock enterprises (3, 4). However, creating healthy herds with genetic improvement programmes in dairy populations takes a long time. Nowadays, it is thought that some genes, such as toll-like receptor genes, could be used to increase immune resistance in livestock.

In mammals, the immune system is divided into the innate and adaptive immune systems. Toll-like receptors (TLRs), an essential component of the immune system, activate innate immune reactions, which then cause adaptive immune

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responses (5, 6). The genes coding the proteins involved in TLRs belong to an ancient gene family that is found in vertebrates, invertebrates, and even plants (7). In mammals, the TLR family plays a crucial role in the recognition of pathogen-associated molecular patterns (PAMPs) in the proteins of pathogenic organisms (8).

The mucosal defence is the most critical part of the innate immune system. Mucosal barriers are the initial line of protection against antigens. They play a critical role in preventing infections. One part of this preventative function is a class of cell surface receptors called 'pattern recognition receptors' (PRRs), which recognise specific PAMPs on bacteria (9). TLRs are the best-known members of the PRR family. After PAMP recognition by TLRs, the innate immune response genes are induced via activation of cellular signalling pathways, including inflammatory cytokines (6). In mammals, there are thirteen members of the TLR family, and each member recognises specific PAMPs (10). Some of the TLRs are located on the cell surface, whereas some parts are intracellular (11). TLR4 is a cell surface receptor that recognises the broad class of PAMPs, and it plays a crucial role in pathogen defence through the activation of innate and adaptive immunity, especially against endotoxins of gram-negative bacteria (9, 12, 13, 14).

TLR4 has an especially important role in the immune recognition of mycobacteria that cause significant breeding diseases (15, 16). The TLR4 gene was mapped in *Bos taurus* chromosome 8 (17). Some mutations in the TLR4 gene may reduce the immune response to microbial cell wall components, including lipopeptides and lipopolysaccharide (15, 16, 18). Due to its role in the development of immunity against bacteria, it has been suggested that the TLR4 gene is a potential candidate gene for resistant-to-breeding diseases, such as mastitis, tuberculosis, brucellosis, paratuberculosis, etc. (10, 19, 20, 21, 22).

A healthy mucosal epithelium and heathly PRRs are the first lines of defence against bacteria in the development of mastitis in the mammary gland (12, 23). Differences in the TLR4 gene expression profile in the mammary gland are associated with mastitis in dairy cattle (23). In this regard, several studies have shown a relationship between TLR4 gene polymorphism and mastitis, somatic cell score (SCS) and lactation persistency in cattle (12, 19, 24, 25). These data demonstrate the importance of polymorphisms in the TLR4 gene and indicate it could serve as a molecular marker for mastitis sensitivity, and might be used in marker-assisted selection (MAS) programmes in dairy herds (19).

Turkey has several native cattle breeds, which are significant not only in terms of genetic resources but also in terms of yields and are described as follows: The Anatolian Black (AB) breed is raised in a broad area in the middle of Turkey for meat. The East Anatolian Red (EAR) breed is reared mostly in Northeast Anatolia primarily for meat, but there is also some milk production. The Turkish Gray (TG) breed is raised in the northwest region of Turkey, again primarily for meat but it is also used for milk production. The South Anatolian Red (SAR) breed is bred in South Anatolia, but it is also raised in Iraq, Syria, Lebanon, and Jordan. It is bred primarily for milk production. The Zavot breed is raised in Northeast Anatolia. It is a hybrid created by crossing Brown Swiss, Simmental, and EAR. It is reared primarily for meat yield. In this context, the objective of the present study was to identify Single Nucleotide Polymorphisms (SNPs) within the TLR4 gene in some Turkish native cattle breeds (Zavot, EAR, AB, SAR, and TG) and Holsteins raised in Turkey.

Materials and methods

Sampling and DNA Extraction

Blood samples were collected from 488 cows, using the tail vein, and placed in sterile vacuumed tubes containing K3 EDTA. They were stored at -20°C until analysis. A total of 488 blood samples were examined from different breeds such as Zavot (n=60, Ardahan), AB (n=59, Ankara and Çankırı), SAR (n=42, Şanlıurfa and Adana), TG (n=60, Edirne and Balıkesir), EAR (n=49, Erzurum and Kars) and Holstein (n=218, Burdur). The extraction of genomic DNA was performed using phenol:chloroform:isoamyl alcohol (25:24:1) and a DNA extraction kit (GeneJET Genomic DNA Purification Kit). Genomic DNA samples were analysed using NanoDrop 2000 (Thermo Scientific) for quantity and quality controls.

This study was approved by the Local Ethics Committees on Animal Experiments at Mehmet Akif Ersoy University (approval no: 03.12.2012/07) and Erciyes University (approval no: 11.12.2013/157).

Table 1: Primer sequence, PCR product size, primer annealing temperature (Ta) and Restriction Endonucleases (RE) (26)

SNPs	Primer (5'-3') Forward, Reverse	PCR	Ta	RE	RFLP Profile
SNP1 (G-1539A)	5'-TTC TTC AAC CCA ACC CAC CT-3' 5'-GCC CTG GCT CAC CAC AAC TA-3'	546bp	59.1°C	*BglI*	AA: 546 GG: 423+123 GA: 546+423+123
SNP2 (G+265C)	5'-GGG TAT TTT GTT ATG GCT GG-3' 5'-CCA TCA TCC TGG CAT TTT-3'	477bp	54.5°C	*Msp*I	GG: 370+65+42 CC: 245+125+65+42 GC: 370+245+125+65+42

Polymorphism Detection and Genotyping

PCR was performed on a total volume of 25 µL consisting of $MgCl_2$ (2 mM for SNP1 and 2.5 mM for SNP2), dNTP (200 µM), primers (5 pmol) (Table 1), 1X buffer, Taq DNA polymerase (1 U/µL), and DNA (~100 ng). Under PCR conditions, there is an initial denaturing step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec. for SNP1 and 32 cycles at 94°C for 30 s for SNP2, 59.1°C for 30 s for SNP1 and 54.5°C for 35 s for SNP2, and 72°C for 45 s for SNP1 and for 30 s for SNP2, with the last cycle at 72°C for 10 min. PCR reactions were performed using the Amplitronyx Series 6 thermal cycler, and amplification products were electrophoresed on 2% agarose gel stained with ethidium bromide. In PCR products, the TLR4 gene was obtained using fast digestive enzymes (Table 1) in accordance with the manufacturer's instructions (Fermentas, Vilnius, Lithuania). The products were electrophoresed on 3% (SNP1) and 4% (SNP2) agarose gel, after which they were imaged using an UV-transilluminator.

Statistical Analysis

Direct calculation was used to predict genetic variants of the TLR4 gene, allele frequencies, and genotype. A chi-square statistic was used to examine whether or not populations were in HWE; other statistical checks were performed using PopGene32 software (27).

Results

In this study, two alleles (A and G) and three genotypes (GG, GA and AA) were examined by digestion of PCR amplification products with the BgII restriction enzyme (Figure 1). Allelic frequency values for Zavot, AB, SAR, TG, EAR and Holstein breeds were identified as 0.44, 0.53, 0.52, 0.57, 0.52, and 0.68, respectively, for the G allele, and 0.56, 0.47, 0.48, 0.43, 0.48, and 0.32, respectively, for the A allele. The GA genotype was the most common genotype in the Zavot, AB, SAR, TG, EAR, and Holstein breeds. All examined breeds were in HWE for TLR4 G-1539A polymorphisms ($p>0.05$).

Two alleles (C and G) and three genotypes (GG, GC and CC) were examined by digestion of PCR amplification products with Mspl restriction enzyme (Figure 2). Allelic frequency values for EAR, SAR, TG, AB, Zavot and Holstein breeds were identified as 0.12, 0.06, 0.29, 0.31, 0.15, and 0.56; respectively for the G allele, and 0.88, 0.94, 0.71, 0.69, 0.85, and 0.44, respectively for the C allele. All examined breeds were in HWE for *TLR4 G+265C* polymorphisms ($p>0.05$).

Discussion

Earlier studies suggested an interaction between TLR gene polymorphisms and greater susceptibility to bacterial infections in different tissues and in different animals (10, 28, 29). It was reported that TLR4 plays a crucial role in the host defence mechanism against gram-negative bacterial and viral agents (30).

It has also been reported that there could be a relationship between TLR4 gene polymorphism and many significant diseases, such as mastitis, brucellosis, and paratuberculosis in cattle (10, 22, 26, 29). Therefore, it is essential to investigate TLR4 gene polymorphism in indigenous cattle breeds. Because indigenous cattle breeds are specific examples of the bovine species, the genetic polymorphism of G+265C and G-1539A SNPs of the TLR4 gene in five Turkish native (EAR, SAR, TG, AB, and Zavot) cattle breeds were investigated. These SNPs are located in the promoter region of the TLR4 gene, and genotypes were detected using

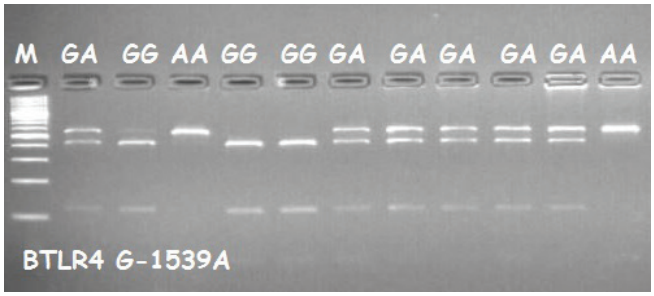

Figure 1: Agarose gel images for *TLR4 G-1539A* polymorphisms. Line M: 100 bp ladder

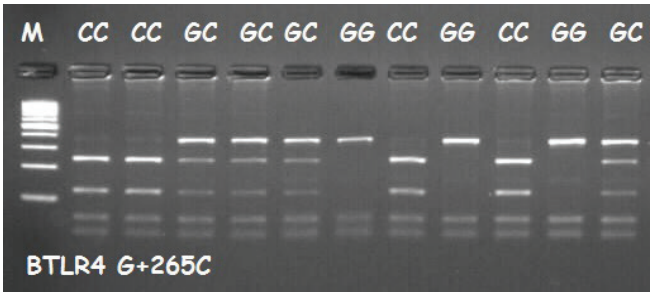

Figure 2: Agarose gel images for the *TLR4 G+265C* polymorphisms. Line M: 100 bp ladder

Table 2: Genotype and allele frequencies of the G-1539A (SNP1) single nucleotide polymorphism in some cattle breeds raised in Turkey

Breed	n	Allele F.		Genotype F. (%)			Heterozygosity		Ne	χ^2 (df=1)
		G	A	GG	GA	AA	Ho	He		
EAR	49	0.52	0.48	28.6	46.9	24.5	0.47	0.50	1.99	0.24 ^{NS}
SAR	42	0.52	0.48	23.8	57.1	19.1	0.57	0.50	1.99	0.75 ^{NS}
TG	60	0.57	0.43	28.3	56.7	15.0	0.56	0.49	1.97	1.27 ^{NS}
AB	59	0.53	0.47	25.4	55.9	18.7	0.56	0.50	1.99	0.78 ^{NS}
Holstein	218	0.68	0.32	44.9	45.9	9.2	0.46	0.44	1.77	0.54 ^{NS}
Zavot	60	0.44	0.56	16.7	55.0	28.3	0.55	0.50	1.97	0.68 ^{NS}

F.: Frequency, Ne: effective number of alleles, NS: Nonsignificant

Table 3: Genotype and allele frequencies of the G+265C (SNP2) single nucleotide polymorphism in some cattle breeds raised in Turkey

Breed	n	Allele F.		Genotype F. (%)			Heterozygosity		Ne	χ^2 (df=1)
		G	C	GG	GC	CC	Ho	He		
EAR	49	0.12	0.88	2.0	20.4	77.6	0.20	0.22	1.27	0.19 ^{NS}
SAR	42	0.06	0.94	0.0	11.9	88.1	0.12	0.11	1.13	0.13 ^{NS}
TG	60	0.29	0.71	8.3	41.7	50.0	0.42	0.42	1.70	0.00 ^{NS}
AB	59	0.31	0.69	8.4	45.8	45.8	0.46	0.43	1.76	0.18 ^{NS}
Holstein	218	0.56	0.44	30.3	51.4	18.3	0.51	0.49	1.97	0.35 ^{NS}
Zavot	60	0.15	0.85	0.0	30.0	70.0	0.30	0.26	1.34	1.75 ^{NS}

F: Frequency, Ne: effective number of alleles, NS: Nonsignificant

PCR-RFLP analysis. These polymorphisms were first reported in Chinese Holsteins, and these polymorphisms could potentially be used as a candidate for the application of molecular marker-assisted selection programmes in Holstein cattle by Li et al. (26).

Li et al. (26) genotyped G+265C and G-1539A polymorphisms in 421 Chinese Holstein cattle, and they identified all three genotypes with different frequencies of two SNPs. At locus G-1539A, which is in the promoter region of the TLR4 gene, the GG genotype frequency was higher (52%) than other genotype frequencies. However, the GA genotype frequency value was higher than other genotypes in five Turkish domestic cattle breeds examined in our study. However, among the cattle breeds examined, the GG genotype frequency (44.9%) was highest in Holstein cattle bred in Turkey. It was also close to the GA genotype frequency (45.9%). An association analysis was performed between the TLR4/G-1539A/BgII polymorphism and 305-day milk yield in Chinese Holstein cattle, and a significant association was detected between 305-day milk yield and the GG genotype. However, the homozygote GG genotype frequency was lower than the GA genotype in all examined cattle breeds. Nevertheless, the five native cattle breeds showed that GA genotype is more frequent than the other two genotype (GG and AA) groups. However, the observed genetic variation continues in the six cattle breeds examined in Turkey. In the light of these results, it can be speculated that increasing the genotype frequency value of the GG genotype at the TLR4/G-1539A locus may be responsible for increasing milk yield in Holstein cattle in Turkey and Turkish native cattle.

For the TLR4/G+265C site, Li et al. (26) found that the frequency of other genotypes in the Chinese Holstein population was lower than the GC genotype. Similar to our results, the GC genotype frequency (51.4%) was higher than other genotypes in the Holstein population raised in Turkey. For this polymorphism, unlike the Holstein breed, we found that the frequency of other genotypes in the five examined Turkish native cattle breeds was lower than the CC genotype. All breeds in the current demonstrated Hardy-Weinberg equilibrium for two SNPs at the TLR4 gene.

Li et al. (26) genotyped this SNP in Chinese Holsteins, and they reported an association between TLR4/G+265C polymorphism and SCS in the examined Holstein population. A previously conducted study reported that it could be a correlation between another SNP named T4CRBR1 in the TLR4 gene and SCS and mastitis in Chinese Holstein cattle (12). They also reported that this correlation was not found in Simmental and Sanhe cattle breeds (12). Another study by this group of researchers found a new SNP, which was an association with SCS in Holstein, Simmental and Sanhe cattle breeds (29). Another study found a relationship between an SNP in the TLR4 gene and bovine brucellosis (22). These investigations show that SNPs in the TLR gene could be used to increase disease resistance in cattle.

The data from this study has demonstrated that TLR4 polymorphisms exist in both Holstein cattle breeds and all indigenous cattle breeds raised in Turkey. Maintaining the variation in the gene pool is essential for genotyping cattle breeds and is a criterion for selection against various infections and development of yield traits in cattle. Further research should be conducted to investigate the relationship between resistance to important breeding diseases, such as mastitis, and yield traits in commonly raised cattle breeds, such as Holstein and native cattle breeds.

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References

1. Jones WP, Hansen LB, Chester-Jones H. Response of health care to selection for milk yield of dairy cattle. J Dairy Sci 1994; 77: 3137–52.

2. Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 2001; 84: 277–93.

3. Egger-Danner C, Willam A, Fuerst C, Schwarzenbacher H, Fuerst-Waltl B. Hot topic: effect of breeding strategies using genomic information on fitness and health. J Dairy Sci 2012; 95: 4600–9.

4. Parker Gaddis KL, Cole JB, Clay JS, Maltecca C. Genomic selection for producer-recorded health event data in US dairy cattle. J Dairy Sci 2014; 97: 3190–9.

5. Takeda K, Akira S. Roles of toll-like recep-

tors in innate immune responses. Genes Cells 2001; 6: 733–42.

6. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev. Immunol 2003; 21: 335–376.

7. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 2006; 117: 979–87.

8. Medzhitov R, Janeway C. Innate immunity. N Engl J Med 2000; 343: 338–44.

9. Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. Immunol Lett 2003; 85: 85–95.

10. Mucha R, Bhide MR, Chakurkar EB, Novak M, Mikula IS. Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium subsp. paratuberculosis* infection in cattle. Vet Immunol Immunopathol 2009; 128: 381–8.

11. Coll RC, O'Neill LA. New insights into the regulation of signaling by Toll-like receptors and Nod-like receptors. J Innate Immun 2010; 2: 406–21.

12. Wang X, Xu S, Gao X, Ren H, Chen J. Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle. J Genet Genomics 2007; 34(5): 406–12.

13. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immun 2001; 2(8): 675–80.

14. Akira S, Takeda KT. Toll-like receptor signaling. Nat Rev Immunol 2004; 4(7): 499–511.

15. Hawn TR, Misch EA, Dunstan, SJ, et al. A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. Eur J Immunol 2007; 37(8): 2280–9.

16. Omueti KO, Mazur DJ, Thompson KS, Lyle EA, Tapping RI. The polymorphism P315L of human Toll-like receptor 1 impairs innate immune sensing of microbial cell wall components. J Immunol 2007; 178: 6387–94.

17. White SN, Kata SR, Womack JE. Comparative fine maps of bovine toll-like receptor 4 and toll-like receptor 2 regions. Mamm Genome 2003; 14(2): 149–55.

18. Schwartz DA. The genetics of innate immunity. Chest 2002; 121: 62S–8S.

19. Sharma BS, Leyva I, Schenkel F, Karrow NA. Association of toll-like receptor 4 polymorphisms with somatic cell score and lactation persistency in Holstein bulls. J Dairy Sci 2006; 89(9): 3626–35.

20. Pinedo PJ, Buergelt CD, Donovan GA, et al. Candidate gene polymorphisms (BoIFNG, TLR4, SLC11A1) as risk factors for paratuberculosis infection in cattle. Prev Vet Med 2009; 91(2/4): 189–96.

21. Pulido I, Leal M, Genebat M, Pacheco YM, Sáez ME, Soriano-Sarabia N. The TLR4 ASP-299GLY polymorphism is a risk factor for active tuberculosis in Caucasian HIV-infected patients. Curr HIV Res 2010; 8(3): 253–8.

22. Prakash O, Kumar A, Sonwane A, et al. Polymorphism of cytokine and innate immunity genes associated with bovine brucellosis in cattle. Mol Biol Rep 2014; 41(5): 2815–25.

23. Goldammer T, Zerbe H, Molenaar A, et al. Mastitis increases mammary mRNA abundance of a-defensin 5, toll-like receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin Vaccine Immunol 2004; 11: 174–85.

24. Zhang LP, Gan QF, Ma TH, et al. Toll-like receptor 2 gene polymorphism and its relationship with SCS in dairy cattle. Anim Biotechnol 2009; 20(3): 87–95.

25. Yuan Z R, Li J, Zhang, LP, et al. Novel SNPs polymorphism of bovine CACNA2D1 gene and their association with somatic cell score. Afr J Biotechnol 2011; 10(10): 1789–93.

26. Li Z, Zhang H, Wang H, et al. Polymorphism in the promoter of TLR4 gene by PCR-RFLP and its association with somatic cell score in Chinese Holstein. Arch Tierzucht 2014; 57(6):1–6.

27. Yeh FC, Yang RC, Boyle T. POPGENE version 1.32: Microsoft Windows–based freeware for population genetic analysis. Edmonton, Alberta, Canada : Center for International Forestry Research, University of Alberta, 2000.

28. Rezazadeh M, Hajilooi M, Rafiei A, et al. TLR4 polymorphism in Iranian patients with brucellosis. J Infect 2006; 53(3): 206–10.

29. Wang XP, Xu SZ, Gao X, Li JY. Cloning and SNP screening of the TLR4 gene and the association between its polymorphism and somatic cell score in dairy cattle. S Afr J Anim Sci 2008; 38(2): 101–9.

30. Mariotti M, Williams JL, Dunner S, et al. Polymorphisms within the toll-like receptor (TLR)-2, -4, and -6 genes in cattle. Diversity 2009; 1: 7–18.

RAZISKOVANJE ENOJNIH NUKLEOTIDNIH POLIMORFIZMOV G+265C IN G-1539A GENA RECEPTORJA TLR4 PRI NEKATERIH PASMAH KRAV, GOJENIH V TURČIJI

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Povzetek: Tolični receptorji oz. receptorji TLR (iz angl. Toll-like receptors) igrajo bistveno vlogo pri reakciji prirojenega imunskega sistema na patogene. Ti receptorji pomagajo prirojenemu imunskemu sistemu prepoznati antigensko strukturo patogenov in sprožiti vnetni odziv. TLR4 je bil izmed skupine toličnih receptorjev prepoznan kot kandidatni molekularni označevalec za odpornost na mastitis pri govedu. V študiji smo želeli opisati G-1539A (BglI) v promotorju in G+265C (MspI) v 5'-UTR polimorfizmih gena TLR4 pri različnih pasmah goveda: pasmi Zavot (n = 60), vzhodno anatolskem rdečem govedu (EAR, n = 49), anatolijskemu črnemu govedu (AB, n = 59), južno anatolskemu rdečemua govedu (SAR, n = 42), turškem sivem govedu (TG, n = 60) in govedu pasme Holstein (n = 218). V ta namen smo uporabili verižno reakcijo s polimerazo in polimorfizem dolžin restrikcijskih odsekov (PCR-RFLP) za genotipizacijo 488 govedi. Z restrikcijsko cepitvijo produktov PCR z restrikcijskim encimom BglI smo pregledali dva alela (A in G) in tri genotipe (GG, GA in AA). Pogostost alela G je bila večja pri vseh raziskanih pasmah, razen pasmi Zavot. Ugotovljeno je bilo, da je genotip GA najpogostejši genotip pri pasmah goveda EAR, SAR, TG, AB, Holstein in Zavot. Dva alela (C in G) in trije genotipi (GG, GC in CC) so bili pregledani z restrikcijsko cepitvijo produkta PCR za SNP G+65C z restrikcijskim encimom MspI. Alel C je bil najbolj razširjen pri vseh pasmah. Vse pregledane pasme so bile znotraj Hardy-Weinbergovega ravnotežja ($p > 0,05$). V raziskavo torej poročamo prvič o obstoju dveh SNP gena TLR4 pri šestih pasmah govedi, vzrejenih v Turčiji. Študija je pokazala, da so pri preiskanih pasmah vidne razlike v teh dveh SNP. Te polimorfizme bi bilo mogoče uporabiti pri prizadevanju za vzrejo čred, odpornih na pomembne bolezni pri govedu, kot je mastitis.

Ključne besede: govedo; PCR-RFLP; promotor; SNP; TLR4

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Original Research Article

PARTICIPATION AND DRUG RESISTANCE OF COAGULASE-POSITIVE STAPHYLOCOCCI ISOLATED FROM CASES OF PYODERMA AND OTITIS EXTERNA IN DOGS

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Abstract: Staphylococcus *pseudintermedius* is considered as a major pathogen in dogs, typically involved in skin and ear infections. Other staphylococci, as well as ß-hemolytic streptococci, *Pseudomonas aeruginosa* or yeast-like fungi of the genus *Malassezia* also play an important role in inflammation. Because of this diversity, an appropriate choice of antimicrobial agent(-s) can be difficult. A total of 474 tests were performed (including 255 pyoderma and 219 *otitis externa cases*). In the case of pyoderma, 82.4% of skin lesions were caused by stap hylococci. Co-infections with ß-hemolytic streptococci (17.3 %), *Malassezia* sp. (15.7 %) and *P. aeruginosa* (4.3 %) were also recorded. For external ear infections, the share of staphylococci in inflammation was lower (44.8 %) than that of *Malassezia* sp. (58 %). Relatively frequent co-infections with ß-hemolytic streptococci (18.8 %) and P. aeruginosa (7.8 %) were also noted. A total of 308 susceptibility tests for coagulase-positive staphylococci were performed (210 and 98 for skin and *otitis externa*, respectively). In ≥ 86 % of cases, amoxicillin potentiated with clavulanic acid, cephalexin and fluoroquinolones effectively inhibited the growth of all bacteria *in vitro*. A total of 25 isolates (24 *S. pseudintermedius* and one *S. aureus*) were considered as methicillin-resistant. The *mecA* gene was identified in 100 % of those strains but only 44 % of the isolates additionally carried the *blaZ* gene. All *mecA*-positive staphylococci were multidrug-resistant, mainly to all ß-lactams, fluoroquinolones, linkozamides, macrolides as well as sulfonamides but always susceptible to mupirocin. Overall, multidrug-resistance varied between 6 % (*otitis externa*) and 9 % (inflamed skin) and may become a significant problem in the future.

Key words: pyoderma; otitis externa; dog; staphylococci; multidrug resistance; *mecA*, mupirocin

Introduction

Staphylococcal infection is one of the most common diseases diagnosed in dogs, since it is commonly seen as a secondary cause of skin changes. Staphylococcal diseases are most often described for multifactorial skin changes throughout the body as various types of purulent dermatitis (pyoderma) (1) or a disease focused on the dog's ear called otitis externa (OE) (2).

Pyoderma occurs in the state of reduced animal immunity or anatomical predisposition (skin folds, etc.) and is often accompanied by other dermatological problems as a complication of the primarily underlying disease: ectoparasites (genera of *Sarcoptes*, *Cheyletiella*, *Demodex*, etc.), ringworm (dermatophytes), allergy (e.g. atopic dermatitis, flea allergic dermatitis or food allergies), internal diseases (mainly hormonal disorders) and sebaceous adenitis (3).

Otitis externa is characterized by inflammation of the external ear canal as a combination of primary factors (parasitic e.g. *Otodectes cynotis*, allergic, etc.), predisposing factors (e.g. ear canal conformation, overproduction of cerumen, moisture, floppy ears, etc.), narrowed ear canals (e.g. hyperplasia, stenosis, foreign bodies, etc.)

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and secondary factors (e.g. bacterial or yeast-like fungi infections, etc.) (4).

Coagulase-negative strains of *Staphylococcus* sp. as well as *Staphylococcus pseudintermedius* are usually normal inhabitants of the dog's skin and mucosa. However, a large number of them in connection with an innate predisposition (e.g. age, breed, steroids, short coat) under favorable environmental conditions for the proliferation causes the appearance of skin inflammation (5). The coagulase test differentiates coagulase-positive strains of *Staphylococcus aureus*, isolates belonging to the *S. intermedius* group (SIG; which is divided into three clusters: *S. intermedius*, *S. pseudintermedius* and *Staphylococcus delphini*) and *S. schleiferi* subspecies *coagulans* from *S. epidermidis* and other coagulase-negative species (6). *Staphylococcus pseudintermedius* is the most commonly isolated staphylococcus in dogs (31 – 68 % of cases, even up to 100% in puppies). *Staphylococcus aureus* and *Staphylococcus schleiferi* subsp. *coagulans* are isolated much less frequently (up to 10 % and 4%, respectively). It appears that these staphylococcal species have evolved separately through adaptation to their respective natural hosts and differ in various aspects concerning ecology, population structure and evolution of antibiotic resistance (7). Additional predispositions for increasing the amount of *S. pseudintermedius* on the skin are breed (8) or atopic dermatitis (9). Gram negative rods of the *Pseudomonas* genus or *Enterobacteriaceae* family (*Escherichia coli*, *Proteus*, etc.) as well as Gram positive environmental rods are definitely less frequently isolated. Among other non-bacterial pathogens, that may be diagnosed in routine cultures, yeast-like fungi (*Malassezia* sp., *Candida* sp., etc.) and dermatophytes (*Microsporum* sp., *Trichophyton* sp., etc.) occur most often (10, 11).

Staphylococcus pseudintermedius and other staphylococci have usually low drug resistance. Then, with a properly selected antimicrobial drug and duration of treatment, anti-inflammatory therapy has a positive effect. Failure in the treatment of pyoderma and/or otitis externa in dogs can be caused by an inadequate selection of the first-line antibiotics (natural resistance), inappropriate dosage (≤ MIC), dose interval and duration or increasing antibacterial resistance to a given antibiotic (after a longer period of administration of the same antibiotic) (12).

The aim of the study was to determine the current participation of coagulase positive staphylococci to the formation of pyoderma and otitis externa in dogs and to estimate the current drug resistance of staphylococci isolates to selected antibacterial agents belonging to various groups of antibiotics, differing in chemical structure and composition.

Material and methods

Microorganisms were isolated from clinical cases of pyoderma and otitis externa (Labo-Wet, Szczecin, Poland), with a particular focus on staphylococci and yeast-like fungi. The research was carried out in the period from January 2017 to December 2018.

The research material consisted of swabs with transport medium or deep skin scrapings (transfer to the growth media within < 24 h). Each sample was cultured on Columbia agar base with 5% defibrinated sheep blood (GRASO Biotech, Starogard Gdański, Poland) as well as some selective media (Mannitol Salt Agar for staphylococci, Edwards Medium (modified) with 7% defibrinated sheep blood for streptococci, MacConkey agar for Gram negative rods and Sabouraud agar supplemented with Tween80 for yeast-like fungi; OXOID, Argenta, Poznań, Poland). The cultures were incubated at +37°C up to 96 h and then evaluated by categorizing bacteria and fungi. Due to the requirement of a longer culture period, the research did not include dermatophytes.

Preliminary division into Gram positive and Gram negative bacteria or yeast-like fungi was determined on the basis of morphological characteristics of the colonies, growth on individual culture media and the Gram staining method with subsequent microscopic examination.

Gram positive catalase-positive cocci capable of rapid growth on Chapman's medium were preliminarily classified as staphylococci. For each isolate, the tube coagulase test (Biomed, Cracow, Poland) was performed to detect free coagulase. In order to preliminarily distinguish *Staphylococcus aureus* from other coagulase-positive staphylococci, a clumping factor - "bound coagulase" test (Staphaurex™ Plus Latex Agglutination Test, Remel, OXOID, Argenta, Poznań, Poland) was performed. Additionally, selected phenotypic traits of coagulase-positive staphylococci were tested: VP test (Voges-Proskauer for acetoin; Mikrolatest®, Erba Mannheim, Brno, Czech Republic), acid production from D-mannitol as well as D-trehalose

(anaerobically) and arginine dihydrolase (ADH) tests (GRASO Biotech, Starogard Gdański, Poland) (13, 14). Growth on chromogenic media (Chromagar *Staphylococcus aureus*, GRASO Biotech Starogard Gdański, Poland), sensitivity to polymyxin B, presence and type of hemolysis as well as colony shape and color determined an additional internal control of staphylococcal differentiation. However, there is no "gold standard" to differentiate phenotypically similar coagulase-positive staphylococci, especially *S. pseudintermedius* and *S. intermedius*. Therefore, a multiplex-PCR (M-PCR) method for species identification of seven coagulase-positive staphylococci (*S. aureus*, *S. intermedius*, *S. pseudintermedius*, *S.delphini* group A and B, *S. schleiferi* subsp. *coagulans*, *S. hyicus*) and one coagulase-negative staphylococcus (*S. schleiferi* subsp. *schleiferi*) was used for all isolated staphylococci strains by targeting the thermonuclease (*nuc*) gene locus according to Sasaki et al. (15). Briefly, a single colony of each strain was suspended in 200 µl of 0.9% NaCl and then the DNA was extracted using the genesig Easy DNA/RNA extraction kit (Primerdesign Ltd, United Kingdom). Supernatants were used as crude DNA extracts for M-PCR. As an internal control of expected band size (electrophoresis on a 1.0% agarose gel), DNA belonging to two reference strains (*S. aureus* ATCC 25923 and *S. pseudintermedius* ED99) was also used.

Gram positive catalase-negative cocci capable of rapid growth on Edwards medium with blood (usually with a strong β hemolysis) were classified as streptococci. To confirm this, a latex agglutination test for the identification of streptococcal Lancefield's groups was used (OXOID, Argenta, Poznań, Poland).

G-negative rods were initially differentiated by growth type on MacConkey agar. Non-lactose fermenting strains were additionally differentiated by the oxidase test (Microbiologics Inc., OXOID, Argenta, Poznań, Poland), while lactose fermenters on ECC chromogenic agar (GRASO Biotech, Starogard Gdański, Poland). For the genus *Pseudomonas*, the ability to produce characteristic odor and dye diffusing into agar was also observed as well as significant drug resistance. Yeast-like fungi were isolated on Sabouraud agar supplemented with Tween80 (GRASO Biotech, Starogard Gdański, Poland).

A detailed analysis of the drug resistance was performed only on staphylococci. Sensitivity to specific groups of antibiotics and antimicrobial agents was tested by the diffusion-disc method according to the VET01-A4 (16) and M100-S27 (17) recommendation. Commercial discs (OXOID, Argenta, Poznań, Poland) used in the present study were saturated with the following antimicrobial agents: amoxicillin with clavulanic acid (20+10 µg), enrofloxacin (5 µg), marbofloxacin (5 µg), ciprofloxacin (10 µg), gentamicin (10 µg), polymyxin B (300 U) trimethoprim with sulphametaxazole (1:19; 25 µg), cefalexin (30 µg), linkomycin with spectinomycin (109 µg), clindamycin (2 µg), neomycin (30 µg) and orbifloxacin (5 µg). For the detection of methicillin resistance, oxacillin (1 µg; resistance with the zone of inhibition ≤ 17 mm recommended for *S. pseudintermedius*; and cefoxitin (30 µg; a surrogate for oxacillin recommended for *S. aureus*, ≤ 21 mm) were used (17). In addition, mupirocin discs (5 µg and 200 µg; OXOID, Argenta, Poznań, Poland; no zone at 200 µg = high level mupirocin resistance) were utilized only in the case of all multidrug-resistant strains (17). Plates of Mueller-Hinton agar (GRASO Biotech, Starogard Gdański, Poland; + 2% of NaCl only for oxacillin) culture medium were used. Incubation was carried out at +35±2 °C (2 x 6 discs on 90 mm plates) and +30±2 °C (only for oxacillin and cefoxitin; testing at temperatures above +35°C may not detect Methicillin-resistant staphylococci) for 18 ÷ 24h. The zones were measured to the nearest millimeter and examined carefully in good light to detect colonies within the zone. In the case of the observed resistance to β-lactam antibiotics, the presence of the *mec*A, *mec*C and *blaZ* genes has also been tested according to Ruzauskas et al. (18).

Results

Over the period considered, a total of 474 samples were examined, including 255 swabs or skin scrapings and 219 swabs from the canine external ear canal. The results are summarized in Tables 1 and 2. In the cases of the S+1, S+2, S+3 and "no STAPH, growth" schemes, particular numbers given for individual pathogens (underlined values) do not refer to the actual number of tests. They only indicate a quantitative contribution in combination with another microorganism.

In the case of pyoderma, the vast majority of skin lesions were caused by staphylococci (82.4%), with more than half of the cases (51%) alone. In the present study, β-hemolytic

streptococci have never been the primary cause of skin lesions. Along with staphylococci, they caused 44 infections (17.3% of cases). Yeast-like fungi were rarely the sole cause of skin changes (only 1.6% of all cases), while their frequent coexistence with staphylococci was found (n = 36, which represented 14.1% of cases) and only one case in mixed infection without staphylococci was observed. "Aseptic" cases accounted for a slightly lower proportion of all samples (12.8%), in which no growth of either bacteria or yeast-like fungi was found despite 96 h of incubation. In these cases, allergies or dermatophytes could not be confirmed or excluded. Of the remaining bacteria belonging to the group of opportunistic microorganisms, the greatest importance was attributed to the *Pseudomonas* spp. (a total of 11 cases; 4.3%).

In the case of *otitis externa*, the share of staphylococci in inflammation was almost half lower (44.8%), of which only 11% of cases of staphylococci presence were the sole cause of changes in the ear (nearly five times less than in the case of skin lesions). On the other hand, the share of yeast-like fungus increased dramatically (58%, almost four times), with up to 26% of the primary cause. It was also the dominant microorganism that complicated wounds together with coagulase-positive *Staphylococcus* sp. (27.4%) or other species (n=10, i.e. 4.6%). Among other microorganisms, the share of the pyogenic rods (*Pseudomonas* sp.) increased significantly. They where present either alone (4.6%) or in mixed growth with staphylococci (3.2%) or other opportunistic bacteria and / or yeast-like fungi (4.6%). β-hemolytic streptococci still constituted a significant proportion of cases (18.8%), sporadically occurring alone (1.4%), but more often with coagulase-positive *Staphylococcus* (11.9%) or other microorganisms (5.5%). In 16% of samples, there was no growth of microorganisms under aerobic conditions after 96 h incubation, which was the basis for ending the test with negative results (marked as "sterile" samples).

Table 1: The participation of staphylococci, yeast-like fungi and other microorganisms in the formation of pyoderma in dogs

type of growth	no growth	Y-L	STAPH	S+1				S+2					S+3					no STAPH, growth:			
				STR	G(-)	G(+)	Y-L	STR	PSE	G(-)	G(+)	Y-L	STR	PSE	G(-)	G(+)	Y-L	PAS	STR	PSE	Y-L
n	33	4	130	19	6	5	21	23	4	8	2	13	2	3	3	2	2	4	4	4	1
				51 (20)				25 (9.8)					4 (1.6)								
(%)	(12.8)	(1.6)	(51)	80 (31.4)														8 (3.2)			
				210 (82.4)																	
total:	255 (100%)																				

S+1, S+2, S+3 *Staphylococcus* growth in addition to one, two or three other microorganisms, respectively n – number of individuals
Y-L yeast-like fungi STAPH - *Staphylococcus* spp. STR - *Streptococcus* spp. PSE - *Pseudomonas* spp. G(-) – Gram negative rods
G(+) – Gram positive rods PAS - *Pasteurella* spp.

Table 2: The participation of staphylococci, yeast-like fungi and other microorganisms in the formation of otitis externa in dogs

type of growth	no growth	Y-L	STAPH	S+1				S+2					S+3				no STAPH, growth:				
				STR	PSE	G(-)	Y-L	STR	PSE	G(-)	G(+)	Y-L	STR	G(-)	G(+)	Y-L	STR	PSE	Y-L	G(-)	G(+)
n	34	58	24 (11)	11	2	1	35	10	3	3	4	20	5	3	2	5	15	20	10	2	2
				49 (22.4)				20 (9.1)					5 (2.3)								
(%)	(16)	(26)		74 (33.8)													29 (13.2)				
				98 (44.8)																	
total:	219 (100%)																				

S+1, S+2, S+3 *Staphylococcus* growth in addition to one, two or three other microorganisms, respectively n – number of individuals
Y-L yeast-like fungi STAPH - *Staphylococcus* spp. STR - *Streptococcus* spp. PSE - *Pseudomonas* spp. G(-) – Gram negative rods
G(+) – Gram positive rods

Table 3: The drug resistance of coagulase-positive staphylococci isolated from pyoderma cases in dogs

antimicrobial agent		coagulase-positive staphylococci (other than *S. aureus*) n = 200*			Total	*Staphylococcus aureus* n = 10			Total
		S	I	R**		S	I	R	
Amoxicillin / Clavulanic acid	n	182	n/a	18	200	9	n/a	1	10
	%	91		9		90		10	
Enrofloxacin	n	172	3	25	200	9	-	1	10
	%	86	1.5	12.5		90	-	10	
Marbofloxacin	n	161	3	22	186	9	-	1	10
	%	86.6	1.6	11.8		90	-	10	
Ciprofloxacin	n	165	3	22	190	9	-	1	10
	%	86.8	1.6	11.6		90	-	10	
Gentamicin	n	148	18	23	189	6	3	1	10
	%	78.3	9.5	12.2		60	30	10	
Polymyxin B	n	166	34	-	200	-	-	10	10
	%	83	17	-		-	-	100	
Trimethoprim with sulfamethoxazole	n	137	21	24	182	9	-	1	10
	%	75.3	11.5	13.2		90	-	10	
Cephalexin	n	158	6	18	182	6	2	1	9
	%	86.8	3.3	9.9		66.7	22.2	11.1	
Clindamicin	n	78	22	46	146	2	1	1	4
	%	53.4	15.1	31.5		50	25	25	
Lincomycin with spectinomycin	n	43	25	13	81	-	2	-	2
	%	53.1	30.9	16		-	100	-	
Neomycin	n	29	10	11	50	2	1	1	4
	%	58	20	22		50	25	25	
Mupirocin	n	18	-	-	18***	1	-	-	1***
	%	100	-	-		100	-	-	
Cefoxitin	n	n/a	n/a	n/a	n/a	9	n/a	1	10
	%					90		10	
Oxacillin	n	182	n/a	18	200	n/a	n/a	n/a	n/a
	%	91		9					

S – susceptible I – intermediate R – resistant n – number of individuals n/a – not applicable
* - S. pseudintermedius n=196; *S. schleiferi* subsp. coagulans n = 4; *S. intermedius* and *S. delphini* were not detected ** - *S. pseudintermedius* in all cases *** - only multidrug-resistant strains

Table 4: The drug resistance of coagulase-positive staphylococci isolated from *otitis externa* cases in dogs

antimicrobial agent		coagulase-positive staphylococci (other than *S. aureus*) n = 96*			Total	*Staphylococcus aureus* n = 2			Total
		S	I	R**		S	I	R	
Amoxicillin / Clavulanic acid	n	90	n/a	6	96	2	-	-	2
	%	93.8		6.2		100		-	
Enrofloxacin	n	87	2	7	96	2	-	-	2
	%	90.6	2.1	7.3		100	-	-	
Marbofloxacin	n	85	2	7	94	2	-	-	2
	%	90.4	2.2	7.4		100	-	-	
Ciprofloxacin	n	82	1	7	90	2	-	-	2
	%	91.1	1.1	7.8		100	-	-	
Gentamicin	n	76	8	8	92	1	1	-	2
	%	82.6	8.7	8.7		50	50	-	
Polymyxin B	n	70	26	-	96	-	-	2	2
	%	72.9	27.1	-		-	-	100	
Trimethoprim with sulfamethoxazole	n	65	5	15	85	2	-	-	2
	%	76.5	5.9	17.6		100	-	-	
Cephalexin	n	85	-	5	90	1	-	-	1
	%	94.4	-	5.6		100	-	-	
Lincomycin with spectinomycin	n	15	8	1	24	-	-	-	0
	%	62.5	33.3	4.2		-	-	-	
Clindamicin	n	10	3	7	20	2	-	-	2
	%	50	15	35		100	-	-	
Neomycin	n	15	10	11	36	1	-	-	1
	%	41.7	27.8	30.6		100	-	-	
Orbifloxacin	n	34	2	9	45	1	-	-	1
	%	75.6	4.4	20.0		100	-	-	
Mupirocin	n	6	-	-	6***	n/a	n/a	n/a	n/a
	%	100	-	-		n/a	n/a	n/a	
Cefoxitin	n	n/a	n/a	n/a	n/a	2	-	-	2
	%					100	-	-	
Oxacillin	n	90	n/a	6	96	n/a	n/a	n/a	n/a
	%	93.8		6.2					

S – susceptible I – intermediate R – resistant n – number of individuals n/a – not applicable
* - S. pseudintermedius n=95; *S. schleiferi* subsp. coagulans n = 1; *S. intermedius* and *S. delphini* were not detected
** - *S. pseudintermedius* in all cases *** - only multidrug-resistant strains

Table 5: Frequency of selected genes among multidrug-resistant strains of coagulase-positive staphylococci

PCR	multi-drug resistant staphylococci (n=25)			
	S. pseudintermedius		*S. aureus*	
	skin	ear	skin	ear
mecA	18/18 (100%)	6/6 (100%)	1/1 (100%)	0 (0%)
total:	24/24 (100%)		1/1 (100%)	
	25/25 (100%)			
mecC	0/18 (0%)	0/6 (0%)	0/1 (0%)	0 (0%)
total:	0/24 (0%)		0/1 (0%)	
	0/25 (0%)			
blaZ	8/18 (44.4%)	2/6 (33.3%)	1/1 (100%)	0 (0%)
total:	10/24 (41.7%)		1/1 (100%)	
	11/25 (44%)			

Among all the above mentioned microorganisms, detailed biochemical and molecular tests were conducted only on staphylococci. In the present study, coagulase-negative staphylococci (including *Staphylococcus schleiferi* subsp. *schleiferi*) occurred very rarely (< 5% of cases during the whole research) and, if detectable, they usually grew as single colonies and for this reason they were ignored in routine research. The vast majority of staphylococci, that were isolated due to their abundant and homogeneous growth, were coagulase-positive (but clumping factor negative), VP-positive, ADH-positive and D-trehalose positive. Mannitol was not fermented (or a weak and delayed fermentation of this sugar occurred; similar observations were made on Mannitol Salt Agar) and always with double-zone hemolysis on sheep blood agar (without pigmentation of colony). All such isolates were sensitive to polymyxin B and grew on Chromagar as mixed violet-blue colonies. After electrophoresis, band size was always at 926 bp (similar to *S. pseudintermedius* ED99 internal control). Taking into account biochemical traits and molecular results (M-PCR), *S. pseudintermedius* was predominant both in the pyoderma (93.33%; Table 3) and *otitis externa* (96.94%; Table 4) cases. Only few β-hemolytic strains (4.76% and 2.04%, respectively) were additionally positive in the clumping factor test, VP-positive, both D-mannitol and D-trehalose positive and ADH-negative (or showed a weak result after 48 h incubation). In addition, they were always resistant to polymyxin B and grew on Chromagar as dark-pink colonies (colony color was in accordance with the recommendations for *S. aureus* given by the manufacturer of the chromogenic media). After M-PCR, all such isolates had a 359-bp amplification fragment similar to the *S. aureus* ATCC 25923 reference strain. These strains were considered to be *Staphylococcus aureus* (Tables 3 and 4). Finally, only 1.91% of the skin and 1.02% of the ear isolates of staphylococci had completely different traits: both sugars were not fermented, while VP and ADH were positive. Colonies on blood sheep agar were β-hemolytic (without pigmentation), clumping factor was negative, sensitivity to polymyxin B was noted and each strain grew on Chromagar as light pink colonies. Without an internal control, band size of M-PCR was estimated at ~ 500 ÷ 550-bp. Both biochemical traits and the size of amplification fragments indicated the presence of *S. schleiferi* subsp. *coagulans*.

For each coagulase-positive *Staphylococcus* isolate, a susceptibility test was carried out for various antimicrobial agents (antibiotics, chemotherapeutics, sometimes with the second component). A total of 308 susceptibility tests were performed, including 210 for skin lesions (Table 3) and 98 for *otitis externa* (Table 4).

In > 90% of cases, regardless of the site from which the *Staphylococcus* strain was isolated, amoxicillin (semi-synthetic β-lactam antibiotic, aminopenicillin) potentiated with clavulanic acid effectively inhibited the growth of these bacteria *in vitro*. Slightly worse activity but also at a high level (86% for the skin isolates and 90 ÷ 91% for the ear isolates), was noted for chemotherapeutics

from the second generation fluoroquinolone group (ciprofloxacin, enrofloxacin and marbofloxacin). However, in 10 ÷ 12% of cases the above-mentioned antimicrobial agents proved ineffective *in vitro*. Another β-lactam antibiotic - cephalexin (first-generation cephalosporin) was also characterized by high efficacy (nearly 87% for the skin and 94% for the ear isolates). Similar results were obtained for other antibiotics: gentamicin (aminoglycoside; 78% and 82% respectively) and one of the polymyxins: Polymyxin B (~ 73% for the ear and 83% for the skin). About 75% of the isolates were sensitive to the third generation of synthetic fluoroquinolone - orbifloxacin (mainly targeted along with antifungal posaconazole for *otitis externa*) and a chemotherapeutic trimethoprim potentiated with sulfametaxazole. The remaining antimicrobial agents were moderately effective.

A total of 24 *S. pseudintermedius* isolates (18 from skin and 6 from ear) were fully resistant to oxacillin (no doubtful results) and considered as MRSP. One *S. aureus* isolate was resistant to cefoxitin and considered as MRSA. This observation was confirmed by PCR tests. Similar to oxacillin and cefoxitin, the *mecA* gene was identified in those 25 out of 308 coagulase-positive strains (8.1 % of total strains) (Table 5; reference strains of *S. pseudintermedius* ED99 and S. aureus ATCC 25923 were *mecA*-negative). Among them, only 44% of the isolates additionally carried the *blaZ* gene encoding penicillinase that is capable of inactivating penicillin(s) by hydrolysis of the beta-lactam ring (19) (*S. pseudintermedius* ED99 was positive and *S. aureus* ATCC 25923 was negative). The rare *mec*C gene has never occurred.

All *mecA*-positive staphylococci were not only to methicillin-resistant but also multidrug-resistant, mainly to all β-lactams, fluoroquinolones, linkozamides, macrolides as well as some antimicrobial agents like sulfonamides. However, these strains were intermediate (predominant) or susceptible to linkomicin with spectinomicin, aminoglycosides and additionally tested tetracyclines. Coagulase-positive staphylococci other than *S. aureus* were always susceptible or intermediate to Polymyxin B. Resistance of dog's *S. aureus* strains to Polymyxin B was confirmed. However, each multidrug-resistant strain was always susceptible to mupirocin and the size of a clear zone of inhibition was always ≥ 20 mm.

Discussion

In the present study, the prevalence of coagulase-positive staphylococci was significantly higher when material was taken from the skin rather than the ear. In the case of pyoderma, the observed proportion of *Staphylococcus* infection was similar to other papers from all over the world. Ruzauskas et al. (18) reported that 76.8% of samples were positive for coagulase-positive staphylococci isolated from sick dogs in Lithuania. In Canada, *S. pseudintermedius* was even isolated from 78% of even healthy dogs (20). Slightly lower prevalence (65.2%) was noted in Portugal (21). In older reports, coagulase-positive staphylococci as well as *S. schleiferi* subsp. *schleiferi* were also isolated from 88% of the cases of inflamed dog's skin (22) and 76.9% of the cases of canine otitis externa (23). In healthy dogs, the carrier prevalence of coagulase-positive staphylococci is estimated at 69% (24) to 87.4% (25).

Besides methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has become a worldwide problem in small animal veterinary medicine (26). Methicillin resistance of both staphylococci is due to the presence of the *mecA* gene which encodes penicillin binding protein 2a (PBP2a) that significantly lowers affinity to all β-lactam antimicrobials (27) and often other classes. The *mecA* gene is located within the chromosome mobile element called the 'staphylococcal chromosomal cassette' (SCCmec). The SCCmec element can be easily transferred between different staphylococcal species (6). Thus, some cases of pyoderma or otitis externa seem to be extremely difficult to treat. Moreover, direct and indirect transmission of *S. pseudintermedius* (including MRSP) may occur between a carrier (even a healthy dog) and humans or other pets (28).

The prevalence of MRSP colonization or contamination has been studied in various dog populations in different countries, with rates of 0–7% in dogs with skin diseases (6). In the present study, the occurrence of *mecA*- and coagulase-positive strains varied between 6 (otitis externa) and 9 % (inflamed skin). In other studies, MRSP was detected in 5/189 (2.6%) (29) and 8/221 (3.6%) (20) of healthy dogs. Griffeth et al. (22) reported that 17% and 8% of affected dogs and 0% and 3% of isolates from healthy dogs were

MRSA and MRSP, respectively. Moreover, most of those MRSP strains also contained a wide range of different antimicrobial drug resistance genes, making them resistant to almost all classes of commonly used antimicrobial agents, even those that are not routinely used to treat staphylococcal infections like amikacin (30). A similar situation was observed in the present study.

Although a few of mupirocin-resistant *S. aureus* strains in human (31) and mupirocin-resistant *S. pseudintermedius* in dogs (32) were isolated, the mechanism of action of mupirocin differs from other clinical antibiotics, rendering cross-resistance to other antibiotics unlikely. In the present study, all multi-drug resistant strains were susceptible to mupirocin, and zones of growth inhibition (> 19 mm at the 5 µg disc) were in agreement with Creagh and Lucey (33). Mupirocin (mixture of several pseudomonic acids) is an antibiotic originally isolated from *Pseudomonas fluorescens* and is structurally unrelated to any other antibiotic (34). Pseudomonic acid is capable of binding to the isoleucyl t-RNA synthetase in *Staphylococcus* sp., resulting in a total inhibition of their protein synthesis (35). Unfortunately, after intravenous or oral administration, mupirocin is rapidly metabolized to monic acid, which has no antibacterial activity. Therefore, only topical treatment of bacterial skin infections (as ointments or creams) or nano-carriers remain available (34).

Conclusions

Routinely used antimicrobial drugs are still effective against the most commonly isolated staphylococci responsible for the development of pyoderma or otitis externa in dogs. However, since 2006, not only MRSA but also MRSP has emerged as a significant animal health problem in veterinary medicine. Such cases (~10%; *mecA*-positive strains), as well as the occurrence of naturally multi-drug resistant *Pseudomonas aeruginosa* (multidrug efflux pumps) or coexistent yeast-like fungi of the genus *Malassezia* (resistance to antibacterial drugs) make initial treatment ineffective and, without the diagnosis of disease etiology, may easily turn into chronic infections. For this reason, clinical cases of pyoderma and otitis externa should be tested more frequently in the laboratory.

References

1. Loeffler A, Lloyd DH. Pyoderma, the march of the staphylococci. Vet Dermatol 2014; 25: 285–6.

2. Dégi J, Imre K, Catana N, Morar A, Sala C, Herman V. Frequency of isolation and antibiotic resistance of staphylococcal flora from external otitis of dogs. Vet Rec 2013; 173: 42.

3. Gross TL, Ihrke PJ, Walder EJ, Affolter VK. Skin diseases of the dog and cat: clinical and histopathologic diagnosis, 2nd ed. Oxford : Blackwell Publishing, 2005: 1–944.

4. Forster SL, Real T, Doucette KP, King SB. A randomized placebo-controlled trial of the efficacy and safety of a terbinafine, florfenicol and betamethasone topical ear formulation in dogs for the treatment of bacterial and/or fungal otitis externa. BMC Vet Res 2018; 14: e262.

5. Löwenstein C. Pyodermie beim Hund [Pyoderma in dogs]. Tierarztl Prax Ausg K Kleintiere Heimtiere 2011; 39: 405–17.

6. Duijkeren van E, Catry B, Greko C, et al. Review on methicillin-resistant *Staphylococcus pseudintermedius*. J Antimicrob Chemother 2011; 66: 2705–14.

7. Bannoehr J, Guardabassi L. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet Dermatol 2012; 23: 253–66.

8. Paul NC, Latronico F, Moodley A, Nielsen SS, Damborg P, Guardabassi L. *In vitro* adherence of *Staphylococcus pseudintermedius* to canine corneocytes is influenced by colonization status of corneocyte donors. Vet Res 2013; 8: 44–52.

9. Min SH, Kang MH, Sur JH, Park HM. *Staphylococcus pseudintermedius* infection associated with nodular skin lesions and systemic inflammatory response syndrome in a dog. Can Vet J 2014; 55: 480–3.

10. Greene CE. Infectious disease of the dog and cat. 4th ed. St. Louis : Elsevier, 2012: 1–1376.

11. Ebani VV, Nardoni S, Bertelloni F, Najar B, Pistelli L, Mancianti F. Antibacterial and antifungal activity of essential oils against pathogens responsible for otitis externa in dogs and cats. Medicines 2017; 4: 21.

12. Patel A, Forsythe PJ. Saunders solutions in veterinary practice: small animal dermatology. Edinburgh, et al. : Saunders, Elsevier Health Science, 2008: 166–8.

13. Sasaki T, Kikuchi K, Tanaka Y, Takahashi N, Kamata S, Hiramatsu K. Reclassification of

phenotypically identified *Staphylococcus intermedius* strains. J Clin Microbiol 2007; 45: 2770–8.

14. Kmieciak W, Szewczyk EM. Coagulase-positive species of the genus *Staphylococcus* – taxonomy, pathogenicity. Post Mikrobiol 2017; 56: 233–44. [in Polish] https://www.exeley.com/advancements_of_microbiology/doi/10.21307/PM-2017.56.2.233 (Dec. 2019)

15. Sasaki T, Tsubakishita S, Tanaka Y, et al. Multiplex-PCR method for species identification of coagulase-positive staphylococci. J Clin Microbiol 2010; 48: 765–9.

16. CLSI. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard. 4th ed. CLSI document VET01-A4. Wayne : Clinical and Laboratory Standards Institute, 2013.

17. CLSI. Performance standards for antimicrobial susceptibility testing. 25th informational supplement. CLSI document M100-S25. Wayne : Clinical and Laboratory Standards Institute, 2017.

18. Ruzauskas M, Couto N, Pavilonis A, et al. Characterization of *Staphylococcus pseudintermedius* isolated from diseased dogs in Lithuania. Pol J Vet Sci 2016; 19: 7–14.

19. Ferreira AM, Martins KB, Silva VR, Mondelli AL, Cunha ML. Correlation of phenotypic tests with the presence of the *blaZ* gene for detection of beta-lactamase. Braz J Microbiol 2017; 48: 159–66.

20. Priyantha R, Gaunt MC, Rubin JE. Antimicrobial susceptibility of *Staphylococcus pseudintermedius* colonizing healthy dogs in Saskatoon, Canada. Can Vet J 2016; 57: 65–9.

21. Beça N, Bessa LJ, Mendes Â, et al. Coagulase-positive *Staphylococcus*: prevalence and antimicrobial resistance. J Am Anim Hosp Assoc 2015; 51: 365–71.

22. Griffeth GC, Morris DO, Abraham JL, Shofer FS, Rankin SC. Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi* in dogs with healthy and inflamed skin. Vet Dermatol 2008; 19: 142–9.

23. Penna B, Varges R, Medeiros L, Martins GM, Martins RR, Lilenbaum W. Species distribution and antimicrobial susceptibility of staphylococci isolated from canine otitis externa. Vet Dermatol 2010; 21: 292–6.

24. Paul NC, Bargman SC, Moodley A, Nielsen SS, Guardabassi L. *Staphylococcus pseudintermedius* colonization patterns and strain diversity in healthy dogs: a cross-sectional and longitudinal study. Vet Microbiol 2012; 160: 420–7.

25. Rubin JE, Chirino-Trejo M. Prevalence, sites of colonization, and antimicrobial resistance among *Staphylococcus pseudintermedius* isolated from healthy dogs in Saskatoon, Canada. J Vet Diagn Invest 2011; 23: 351–4.

26. Grönthal T, Eklund M, Thomson K, Piiparinen H, Sironen T, Rantala M. Antimicrobial resistance in *Staphylococcus pseudintermedius* and the molecular epidemiology of methicillin-resistant *S. pseudintermedius* in small animals in Finland. J Antimicrob Chemother 2017; 72: 1021–30.

27. Detwiler A, Bloom P, Petersen A, Rosser EJ, Jr. Multi-drug and methicillin resistance of staphylococci from canine patients at a veterinary teaching hospital (2006–2011). Vet Q 2013; 33: 60 –7.

28. Bajwa J. Canine superficial pyoderma and therapeutic considerations. Can Vet J 2016; 57: 204–6.

29. Kjellman EE, Slettemeås JS, Small H, Sunde M. Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) from healthy dogs in Norway - occurrence, genotypes and comparison to clinical MRSP. MicrobiologyOpen 2015; 4: 857–66. doi: 10.1002/mbo3.258

30. Gold RM, Cohen ND, Lawhon SD. Amikacin resistance in *Staphylococcus pseudintermedius* isolated from dogs. J Clin Microbiol 2014; 52: 3641–6.

31. Cookson BD. The emergence of mupirocin resistance: a challenge to infection control and antibiotic prescribing practice. J Antimicrob Chemother 1998; 41: 11–18.

32. Matanović K, Pérez-Roth E, Pintarić S, Šeol Martinec B. Molecular characterization of high-level mupirocin resistance in *Staphylococcus pseudintermedius*. J Clin Microbiol 2013; 51: 1005–7.

33. Creagh S, Lucey B. Interpretive criteria for mupirocin susceptibility testing of *Staphylococcus* spp. using CLSI guidelines. Br J Biomed Sci 2007; 64: 1–5.

34. Opatrilova R, Jampilek J. Rapid screening of mupirocin skin permeation modification by micronized and nanonized alaptide. ADMET & DMPK 2014; 2: 56–62. doi:10.5599/admet.2.1.26

35. Hughes J, Mellows G. On the mode of action of pseudomonic acid: inhibition of protein synthesis in *Staphylococcus aureus*. J Antibiot 1978; 31: 330–5.

VKLJUČENOST IN ODPORNOST NA ANTIBIOTIKE STAFILOKOKOV, POZITIVNIH NA KOAGULAZO, IZOLIRANIH IZ PRIMEROV PIODERME IN VNETJA ZUNANJEGA UŠESA PRI PSIH

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Povzetek: Bakterijea *Staphylococcus pseudintermedius* so ene od pomembnejših patogenih bakterij pri psih in so običajno prisotne pri okužbah kože in ušes. Pomembno vlogo pri teh vnetjih pa imajo običajno tudi drugi stafilokoki, ß-hemolitični streptokoki, *Pseudomonas aeruginosa* ali kvasovkam podobne glive iz rodu *Malassezia*. Zaradi raznolikosti povzročiteljev vnetja je primerna izbira protimikrobnih zdravil lahko težavna. V študiji je bilo skupno opravljenih 474 testov (vključno z 255 primeri pioderme in 219 primeri vnetja zunanjega ušesa). V primeru pioderme so 82,4 % kožnih vnetij povzročili stafilokoki. Zabeležene so bile tudi sočasne okužbe z ß-hemolitičnimi streptokoki (17,3 %), *Malassezia* sp (15,7 %) in *P. aeruginosa* (4,3 %). Pri okužbah zunanjih ušes je bil delež stafilokokov v vnetjih nižji (44,8 %) kot pri *Malassezia sp.* (58 %). Opažene so bile tudi sorazmerno pogoste sookužbe z ß-hemolitičnimi streptokoki (18,8 %) in *P. aeruginosa* (7,8 %). Opravljenih je bilo skupno 308 testov občutljivosti za stafilokoke, pozitivne na koagulazo (210 za kožo in 98 za vnetje zunanjega ušesa). V manj kot 86 odstotkov primerov je amoksicilin, z dodatkom klavulanske kisline, cefaleksina in fluorokinolonov *in vitro* učinkovito zaviral rast vseh bakterij. Skupaj 25 izolatov (24 *S. pseudintermedius* in 1 *S. aureus*) je veljalo za odporne proti meticilinu. Gen *mecA* je bil identificiran v vseh sevih, vendar je le 44 % izolatov imelo gen *blaZ*. Vsi *mecA*-pozitivni stafilokoki so bili odporni na več zdravil, večinoma na vse ß-laktame, fluorokinolone, linkozamide, makrolide in sulfonamide, vendar so ostali dovzetni za mupirocin. Na splošno je odpornost na več zdravil znašala med 6 % (vnetje zunanjega ušesa) in 9 % (vnetje kože), kar lahko v prihodnosti povzroča velik problem pri zdravljenju tovrstnih okužb.

Ključne besede: pioderma; vnetje zunanjega ušesa; pes; stafilokoki; odpornost na več zdravil; *mecA*; mupirocin

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