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DETECTION AND ANTIMICROBIAL RESISTANCE OF COAGULASE-POSITIVE *STAPHYLOCOCCUS AUREUS* AND METHICILIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM RAW PORK IN THE RETAIL

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is widespread in pork meat and can cause a severe food poisoning. The aim of present study is to determine the prevalence and characteristics of the antimicrobial resistance of *S. aureus* and MRSA from retail raw pork meat in Bulgaria between 2019 and 2021. Coagulase-positive *S. aureus* were detected in 30.8% (52/169). Susceptibility to all antimicrobials was detected in 28.8% (15/52) of *S. aureus* isolates by the disk-diffusion method. Multidrug resistance was found in 18 of a total of 52 isolates (34.6%). A study of the MIC indicated resistance to the cefoxitin (> 4 mg/L) in 8 of the total 23 tested isolates (34.8%) The *mecA* gene was confirmed in 5 of the tested isolates. The high prevalence of MRSA in raw pork meat, sold in Bulgaria should be considered as a signal of the risk of its spread in the human population. This requires the implementation of appropriate hygiene practices in the production, processing and sale of meat and meat products to reduce the spread of MRSA to humans.

Key words: antimicrobial resistance, MRSA, pork, multidrug resistance, PCR.

Introduction

S. aureus is the most important human pathogen of the staphylococcal group with clinical symptoms varying from local skin infection to severe, often life-threatening infections (Lowy 1998). *S. aureus* possesses strong pathogenic properties as a result of the presence of a large number of different virulence factors. Additionally, *S. aureus* have a tendency in most cases to develop resistance to antimicrobials (Lowy 1998). Resistance to β-lactam antibiotics, known as methicillin resistance, is most commonly. Methicillin-resistant *S. aureus* (MRSA) has been shown to be resistant to almost all antibiotics in this widely used group that are still used in both human and veterinary medicine. Most animals are colonized with *S. aureus*, but only a few are MRSA and are usually isolated from livestock such as pigs, cows, chickens, etc. (De Neeling et al. 2007).

The worldwide use of antimicrobials in the treatment of ubiquitous bacterial infections has caused the development of antimicrobial resistance. Antimicrobials are used in human and veterinary medicine, which allows the transmission of resistant bacteria from animals to humans. Transmission occurs not only through contact with animals but also through consumption of food of animal origin (Bywater et al. 2004).

S. aureus that develop resistance to β-lactam antibiotics are included in the group of methicillin-resistant *S. aureus* (MRSA). MRSA has been shown to be resistant to almost all antimicrobials in this group. MRSA was identified for the first time in 1961 in American hospitals (Barber 1961). Thereafter, it became detected with increasing frequency in hospital isolates worldwide. MRSA was

isolated from cows with mastitis in Belgium almost ten years later, in 1972 (Devriese and Hommez 1975) and gradually became a worldwide pathogen (Goudarzi et al. 2017).

Lakhundi and Zhang (2018) summarized the data on the relationship between livestock and the prevalence of MRSA and concluded that the origin of MRSA is most commonly associated with swine. Van Duijkeren et al. (2008) also demonstrated that pigs are a major risk factor for the spread of MRSA in the Netherlands. In 2006, they analysed different types of pig farms and found that 23 % of them were positive for MRSA. Furthermore, 5 out of 6 breeding farms, which supply fattening farms with piglets, are also positive for MRSA. The presence of MRSA positive workers in all positive farms demonstrates the possibility of MRSA transmission from animals to humans.

Materials and methods

Sample collection

A total of 169 samples of retail packaged raw pork meat were randomly selected from the retail network in Bulgaria between August 2019 and December 2021. The samples were of different origins as follows: Bulgaria (n=123), Belgium (n=14), Spain (n=15), Germany (n=2), Hungary (n=1), France (n=1) and of unknown (n=13). Each sample in a individual package weighing at least 200 g. The samples were transported to the laboratory at 4°C and tested immediately after receipt.

Isolation of S. aureus

The presence of coagulase-positive staphylococci in 1 g of the tested sample was carried out according to ISO 6888-3+AC (2005). Biochemical identification was done with API®STAPH (BioMerieux, France) following the manufacturer's instructions.

Antibiotic susceptibility testing

52 isolates of coagulase-positive *S. aureus* were tested for susceptibility to Ampicillin (AMP, 2 µg), Penicillin (P, 1 UI), Cefoxitin (FOX, 30 mg), Ciprofloxacin (CIP, 5 µg), Oxacillin (OX, 1 µg), Clindamycin (CD, 2 µg), Erythromycin (E, 15 µg) and Chloramphenicol (C, 30 µg) by the disc diffusion method, according to the EUCAST standardised disc diffusion method instructions (EUCAST 2021, Humphries et al. 2018). The reporting of the results was done with measured of the growth inhibition zone. We reported the result in mm, and interpretations were based on the protocol described in CLSI (2020).

Method for determining the minimum inhibitory concentration (MIC)

Isolates showing resistance to at least two antimicrobial classes by the disk diffusion method (n=23) were checked using the microdilution assay according to the CLSI method for detection of the minimal inhibitory concentration (MIC) (CLSI 2020). We used the Sensititre Staphylococci plate – EUST (Thermo Trek Diagnostics, OH, USA) and prepared the test according to ISO 20776-1 (2019).

The antimicrobial agents used in the MIC test and the interpretation ranges are given in Table 1.

Table 1: List of antimicrobial agents, their subclasses, scope of testing and interpretation of sensitivity results.

Antimicrobial sub-classes	Antimicrobial agents	Scope of testing (mg/l)	Interpretation		Source
			S ≤	R >	
Macrolides	Clindamycin (CLI)	0.12-4	0.25	**0.5**	EUCAST,2021
	Erythromycin (ERY)	0.25-8	1	**2**	EUCAST,2021
Tetracyclines	Tetracycline (TET)	0.5-16	1	**2**	EUCAST,2021
Anzamycin	Rifampin (RIF)	0,016-0,5	0,06	**0,5**	EUCAST,2021
Aminoglycosides	Streptomycin (STR)	4-32	16	**16**	EUCAST,2021
	Kanamycin (KAN)	4-64	8	**8**	EUCAST,2021
Penicillins	Penicillin (PEN)	0,12-2	0,125	**0,125**	EUCAST,2021
Phenicols	Chloramphenicol (CHL)	4-64	8	**8**	EUCAST,2021
Pleuromutilins	Tiamuline (TIA)	0,5-4	2	**2**	EUCAST,2021
Streptogramins	Quinupristin/Dalfopristin (SYN)	0,5-4	1	**2**	EUCAST,2021
Glykopeptide	Vancomycin (VAN)	1-16	2	**2**	EUCAST,2021
Aminoglycoside	Gentamycin (GEN)	1-16	1	**1**	EUCAST,2021
Fluoroquinolone	Ciprofloxacin CIP	0,25-8	0,001	**1**	EUCAST,2021
Cephalosporins	Cefoxitin (FOX)	0,5-16	4	**4**	EUCAST,2021
Oxazolidinones	Linezolid (LZD)	1-8	4	**4**	EUCAST,2021
Pseudoamonium acid	Mupirocin (MUP)	0,5-256	1	**1**	EUCAST,2021
Miscellaneous agents	Fusidat (FUS)	0.25 – 4	1	**1**	EUCAST,2021
	Trimethoprim (TMP)	2-31	4	**4**	EUCAST,2021
	Sulfamethoxazole (SMX)	64-512	128	**128**	EUCAST,2021

Molecular Analysis

The confirmation of MRSA was carried out using the multiplex polymerase chain reaction (PCR) method, which can be used to confirm methicillin resistance by amplification of the *mecA*/*mecC* genes, and identification of *S. aureus* by amplification of the *spa* gene (also used for typing) and detection of the gene encoding Panton Valentin Leukocidin (*PVL* or *LukF PV*) (Stegger et al. 2012).

PCR amplification was optimized in a 25 µl reaction mixture containing 2 µl of isolated DNA and 23 µl of mastermix (6.5 µl PCR H2O, 12, 5 µl 2xGreen PCR MasterMix, 2 µl Primer Mix 1 (containing 10 pmol each of *spa*-1113F, *mecA* P4, *pvl-F* and *mecA*LGA251 MultiFP), 2 µl Primer Mix 2 (containing 10 pmol each of *spa*-1514R, *mecA* P7, *pvl-R*, *mecA*LGA251MultiRP). The polymerase chain reaction was carried out in a C1000 TouchTM ThermalCycler (BioRad, USA) by initial denaturation at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 30 s, extension at 58℃ for 1 min and at 72℃ for 1 min, and separation at 72℃ for 10 min.

Amplified DNA fragments (Table 2) were visualized after horizontal electrophoresis in a 2% agarose gel (SeaKem Agarose) in 1xTAE-buffer at 130 V for 30 min. Gel coloration was performed in ethidium bromide solution for 20 min. The results were visualized with UV light (>2500 µW/cm3). GeneRuler 100bp DNA Ladder (Fermentas) was used as a 100 bp marker to determine molecular weight.

Table 2: Nucleotide sequence of primers and amplicon size

Target gene	Name of the primers	Nucleotide sequence (5' - 3')|	Amplicon size
mecA	mecA P4	TCCAGATTACAACTTCACCAGG	162 bp
	mecA P7	CCACTTCATATCTTGTAACG	
spa	spa-1113F	TAAAGACGATCCTTCGGTGAGC	180-600 bp
	spa-1514R	CAGCAGTAGTGCCGTTTGCTT	
PVL	pvl-F	GCTGGACAAAACTTCTTGGAATAT	85 bp
	pvl-R	GATAGGACACCAATAAATTCTGGATTG	
mecC	mecA $_{LGA251}$ MultiFP	GAAAAAAAGGCTTAGAACGCCTC	138 bp
	mecA$_{LGA251}$ MultiRP	GAAGATCTTTTCCGTTTTCAGC	

All isolates positive for the *mecA*/*mecC* gene were identified as MRSA.

Statistical data analysis

The statistical significance of differences between proportions was evaluated with Chi-square ($\chi 2$) tests using Epitools (Sergeant 2018).

Results

Prevalence of S. aureus and MRSA from retail pork meat

Of total 169 raw pork samples, 52 (30.7%) were positive for coagulase-positive *S. aureus*. Coagulase-negative staphylococci were detected in 53.8% of the samples (91 out of 169), and the absence of staphylococci was found in only 26 out of 169 raw pork samples (15.4%).

The prevalence of *S. aureus* in raw pork samples, depending on their origin is presented in Fig. 1.

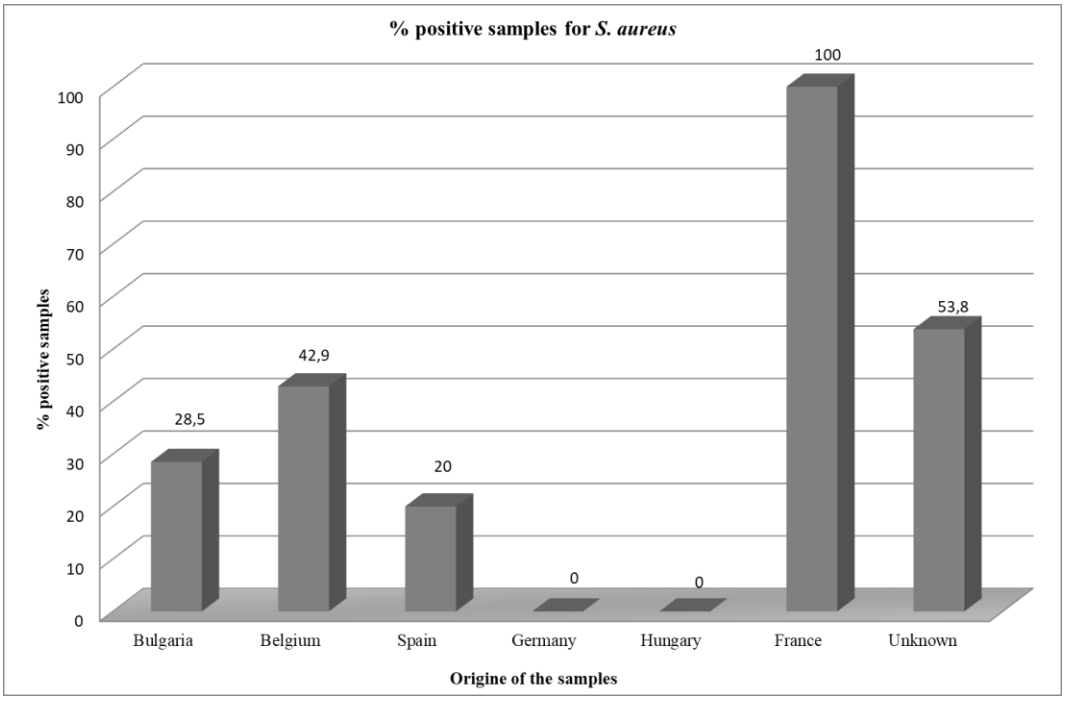

Figure 1: Presence of *S. aureus* in raw pork samples, depending on their origin.

Analysis of the results showed *that S. aureus* was detected in 35 out of 123 samples (28.5%) originating from Bulgaria. The highest prevalence of *S. aureus* in raw pork meat was demonstrated in samples with unknown origine (7 out of 13, 53.8%), from Belgium (6 out of 14, 42.9%), followed by those originating from Bulgaria (35 out of 123, 28.5%) and Spain (3 ot of 15, 20.0%). *S. aureus* was not detected in any of the tested samples from Germany and Hungary. The results for the pork samples, originated from France (1 out of 1, 100.0%) were not statistically significant.

MRSA was detected in 8 of total 169 raw pork meat samples (4.7%). All samples positive for MRSA originated from Bulgaria. MRSA is included in the number of positive *S. aureus*.

Antimicrobial resistance of coagulase-positive S. aureus isolates by disk-difussion method

Susceptibility to all antimicrobials was detected in 15 out of total 52 *S. aureus* isolates (61.5%). Furthermore, the antimicrobial susceptibility profile of the 52 tested isolates by the disc diffusion method showed that resistance to P was the most common (63.5%), followed by resistance to AMP (50.0%), CIP (28.8%), and OX (19.2%) (Fig. 2).

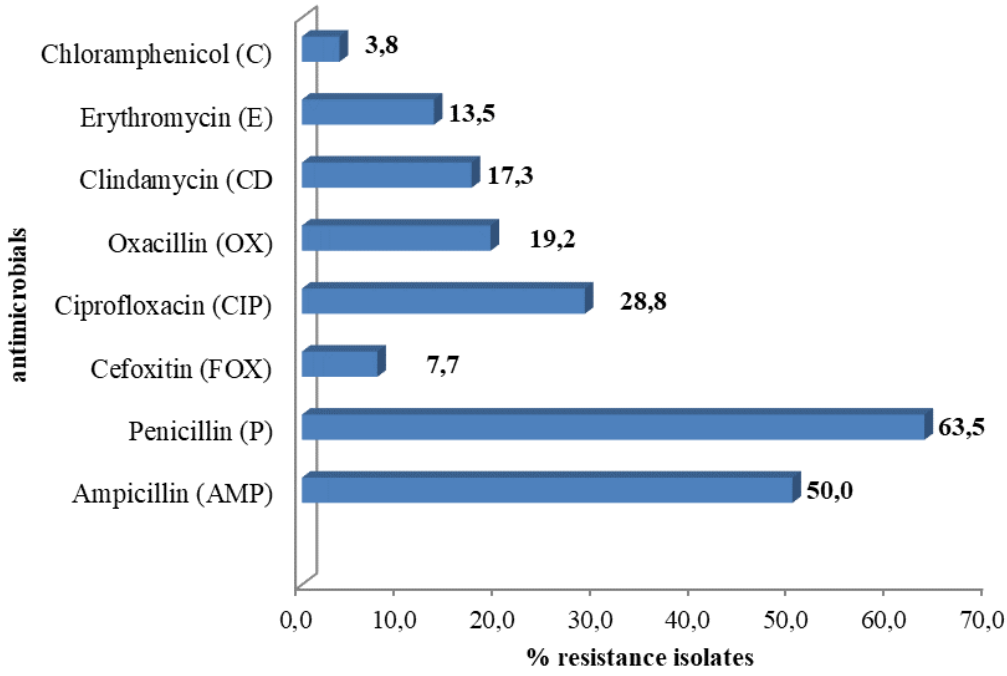

Figure 2: Antimicrobial resistance of *S. aureus* isolates, confirmed by the disc-diffusion method.

Resistance to cefoxitin was found in 4 out of total 52 *S. aureus* isolates (7.7%), which classified them as methicillin-resistant. Multidrug resistance (resistance to >3 antimicrobial classes) was observed in 18 out of total 52 isolates (28.8%).

Antimicrobial resistance of S. aureus isolates by MIC method

The tested isolates *S. aureus* showed different percentages of resistance to the different antibiotics (Fig. 3). All isolates *S. aureus* were resistant to SMX (100%) with values ≥512 mg/l with a cut-off value >128 mg/l. The highest resistance was observed to TET (60.9%), followed by PEN

(52.2%) and STR (26.1%), while the lowest resistance was found to VAN and LZD (4.3%). Multidrug resistance (resistance to more than 3 classes of antibiotics) was found in 15 out of 23 isolates tested (65.2%).

Two out of a total 23 (8.7%) pork isolates showed multidrug resistance to 16 out of 19 antibiotics tested.

Eight out of 23 (34.8%) *S. aureus* isolates showed resistance to cefoxitin >4mg/l, which were identifiedas methicillin-resistant *S. aureus* (MRSA).

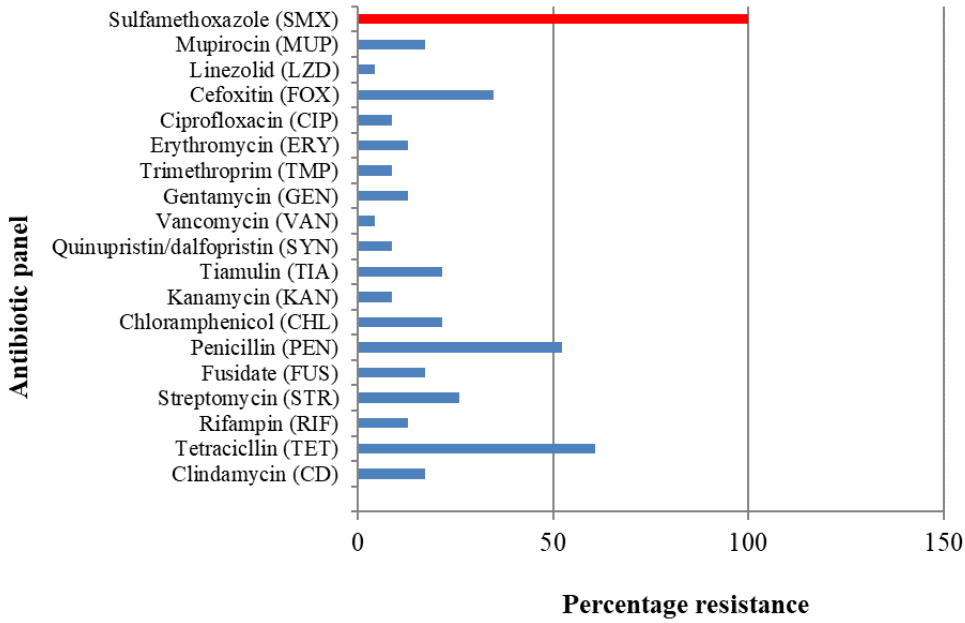

Figure 3: Overall percentage resistance of *S. aureus*, confirmed by the MIC method

PCR Detection of mecA/mecC genes of S. aureus isolates

Methicillin-resistant *S. aureus* (MRSA) genes (*mecA*/*mecC*) were identified in 5 out of total 8 isolates (62.5%) (Fig. 4).

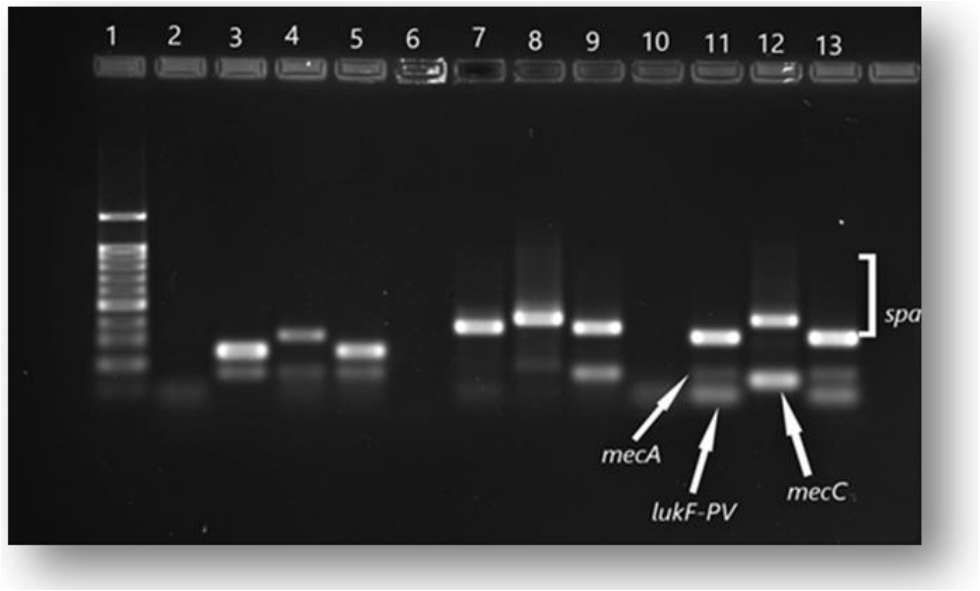

Figure 4: Detection of the *mecA*/*mecC* genes by polymerase chain reaction in pork isolates showing resistance to methicillin by the MIC method.

LEGEND: 1 - DNA loader, 100 bp; 10-negative control; 11 and 13 - PVL-positive control possessing spa (180-600 bp), lukF-PV (85 bp) and mecA (162 bp) genes; 12 - MRSA-positive control possessing spa (180-600 bp) and mecC (138 bp) genes; 3 ÷ 5; 7-9 - MRSA-positive isolates from chilled pork; 2 and 6 MRSA-negative isolates

Two of the swine isolates carrying methicillin resistance genes also showed multi-drug resistance (to 16 of the 19 antibiotics tested). It is interesting to note that some isolates were found to be resistant by the MIC method without this being confirmed by the presence of the responsible genes. This is most likely due to the fact that the primer pairs used were only for the major resistance genes.

The *lukF*-PV gene was only detected in 1 isolate of raw pork meat.

Discussion

For the first time in Bulgaria, a study that provides complex information on the prevalence of *S. aureus*, its antimicrobial resistance, the prevalence of methicillin-resistant *S. aureus* and the presence of resistance genes in the isolates from retail pork from different origins has been conducted.

The prevalence of *S. aureus* in raw pork meat retail in Bulgaria was 30.7%. Analysis of the available scientific data showed a similar prevalence of *S. aureus* in pork samples. Similar to our results in Georgia, a prevalence of *S. aureus* was reported in 45% of the pork samples (Jackson et al. 2013). Kim et al. (2020) also detected S. *aureus* in 20.9% of pork samples in Korea. In contrast to our data, they reported 20.9% positive for *S. aureus* in imported pork samples and 15.1% positive in domestic pork samples.

In contrast to our data, in Denmark was found a significantly higher prevalence of *S. aureus* in pork (60%) (Tang et al. 2017). Similar data were found in USA and showing the presence of *S. aureus* in 64.8% of pork samples. However, the authors reported no significant difference between

conventionally produced meat and from pigs declared free of antibiotics and other growth promoters during fattening (O'Brien et al. 2013).

Antibiotic resistance of *S. aureus* can be transmitted through contaminated meat to consumers (Hanson et al. 2011; Kelman et al. 2011). In our study, *S. aureus* isolates showed the highest resistance to penicillin and tetracycline, above 50.0% both the disk diffusion method and the minimal inhibitory concentration method. This is not unexpected as these two antibiotics have a broad spectrum of action and are very commonly used to treat a variety of bacterial infections in animals and humans. Similar highly expressed resistance to ampicillin and penicillin has been reported by other authors (Jackson et al. 2013; Wu et al. 2019). We also found high sensitivity to chloramphenicol, 96.2% of the isolates were sensitive to it. Similar results were reported by other authors, who identified chloramphenicol as the antimicrobial with the lowest resistance among *S. aureus* (Wu et al. 2019). Some authors report 100% susceptibility to the chloramphenicol (Kalupahana et al. 2019; Tang et al. 2017). The most likely reason for this is that this antimicrobial agent has not been used for treatment in humans and animals in recent years.

The resistance to SMX by the minimum inhibitory concentration method was 100%. In contrast, other authors have reported resistance between 3.5% and 20.0% (Mahros et al. 2021; O'Brien 2012; Wu et al. 2019). Jackson et al. (2013) tested 57 isolates from commercially sold pork meat and found a total susceptibility to sulfamethoxazole. Compared to other studies, some differences in results may also be due to the animal population, inappropriate antibiotic use, and the use of antibiotics as a growth factor included in feed.

Multi-drug resistance (MDR) detected in isolates from pork meat is described as resistance to 3 or more classes of antibiotics. In our study, MDR was present in 65.2% of *S. aureus* isolates by MIC method. These results indicate that multidrug-resistant *S. aureus* are present in pork and can be a transmission risk to humans and cause infections that may be difficult to treat. Velasco et al. (2022) reported similarly worrying data.

Methicillin-resistant *S. aureus* (MRSA) is a serious public health problem. Mortality rates due to MRSA infections have remained high in recent years. In some Asian countries such as Taiwan, China, Japan and South Korea the prevalence of MRSA in hospitals reaches up to 70–80% (Chuang and Huang 2015). The situation among the clinical isolates in Bulgaria is also alarming – 33.6% (Bozhkova 1999). In addition to the prevalence of *S. aureus*, the percentage of MRSA in our samples (4.7%) is similar to other studies. MRSA in pork sold at retail ranged from 0.14% to 6.6% (Hanson et al. 2011; Pu et al. 2009). Weese et al. (2010) also reported a significantly lower prevalence of MRSA in pork, with only 9.6% of the samples. In contrast, Tang et al. (2017) reported up to 15% prevalence of MRSA in pork meat.

The gene encoding resistance to β-lactam antimicrobials (*mecA*/*mecC*) in *S. aureus* was detected by polymerase chain reaction (PCR). We detected the presence of *mecA*/*mecC* in 5 out of 8 isolates (62.5%) showing MIC to cefoxitin >4 mg. Similar results were obtained by Abdel-Moein et al. (2019), who detected the *mecA* gene in 80% of the isolates. In contrast, Yahya et al. (2021) found the *mecA* gene in only 17% of the isolates. Other studies carried out in Korea, China, Brazil and the USA found the presence of the *mecA* gene in 0.7%, 7.9% and 6.6%, respectively (Kim et al. 2020; O'Brien et al. 2012; Rizek et al. 2011). Its presence was also reported in 42.5% of the *S. aureus* strains examined in a study conducted in Bulgaria in 2011 (Gogov 2011).

The presence of *lukF-PV* was detected in only 1 methicillin-resistant isolate, which was fully consistent with previous studies in which the authors also found it rarely (Pu et al. 2009; Wu et al. 2019).

The difference in the results may be explained by the various sources of the samples, as well as the use of different molecular techniques in the countries to detect the *mecA* gene product. However, the consistency in the prevalence of MRSA in pork meat provides a reason to suggest that all these differences may not interfere with the comparison between studies.

Conclusion

It can be concluded that the existing antibiotic resistance present in isolates from raw pork meat poses a risk to consumers. The presence of cefoxitin-resistant *S. aureus* that are *mecA*-negative suggests other mechanisms of transmission of methicillin resistance. This should guide the implementation of strong policies and strategies for antibiotic use in prevention and treatment, both in humans and animals. Limiting their use and appropriate controll of their use in agriculture and food production is a first step in achieving the fight against antimicrobial resistance.

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