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Phenotypic and genotypic study of resistance to Zinc and Cadmium salts in methicillin-resistant *Staphylococci* isolated from humans and domestic animals

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Abstract

The aim of this study was to investigate the emergence of staphylococci resistant to heavy metals among methicillin-resistant staphylococcus spp. (MRS) for the first time in Iran. In the present study, in the first step, 300 staphylococcus isolates from humans and domestic animals (cows, horses, dogs, and cats) were studied by disk diffusion method to identify resistance to methicillin. Then among the resistant isolates, *Staphylococcus aureus* (*S. aureus*) was confirmed by detecting the *nucA* gene using PCR. After identifying the MRS and methicillin-resistant *S. aureus* (MRSA) strains, these isolates were phenotypically tested to evaluate the resistance to zinc chloride and cadmium acetate using Muller Hinton agar (MHA) culture medium supplemented with metal. Finally, *mecA* and *czrC* genes were examined using the PCR method to evaluate the genotypic resistance to methicillin and heavy metals, respectively. The frequency of phenotypic MRS isolates in the phenotypic evaluation was 51 (17%), and almost half of the MRS isolates (n = 25 or 49%) were confirmed to be MRSA using the *S. aureus* -specific PCR assay for the *mecA* gene. Heavy metal susceptibility testing using Mueller Hinton agar plates (Becton Dickinson) supplemented with either zinc chloride or cadmium acetate revealed that 49% and 8% of MRS isolates were resistant to zinc chloride and cadmium acetate, respectively. PCR of the *czrC* gene showed that only 66.7% of dog MRS isolates phenotypically resistant to these heavy metals harbored the *czrC* gene. Here, phenotypic resistance to methicillin had a significant relationship with the existence of the *mecA* gene in all MRS strains. Interestingly, in dog MRS strains the phenotypic resistance to heavy metals had a significant relationship to the harboring of the *czrC* gene, and also there was a high correlation between *mecA* and *czrC* genes. Therefore, the presence of heavy metals (zinc and cadmium) in the living environment of staphylococci makes these bacteria resistant to methicillin.

Introduction

The genus Staphylococcus is Gram-positive cocci and belongs to the family Staphylococcaceae. Although staphylococci are normally commensal bacteria in humans and animals, they can cause a variety of diseases and infections including food

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poisoning, impetigo, cellulitis, and mastitis (1-5). Penicillin was the choice antibiotic to treat patients with staphylococcal infections, but unfortunately, after a decade, some strains of S. aureus became resistant to penicillin via the production of an enzyme named β-lactamase (PBP2a). Methicillin, a semi-synthetic penicillin, was developed in 1959 and was effective against staphylococcal infections, but later on, some S. aureus isolates became resistant to methicillin and a strain called methicillin-resistant Staphylococcus aureus (MRSA) emerged (1, 6). Other studies in Iran and other countries show that the frequency of MRS is variable and mostly ranges from 21% to 61% (7-10).

Like all living organisms, as a result of selective pressure, antibiotic resistance has emerged to this vital antimicrobial agent (1). One of the main reasons for the resistance of some isolates of staphylococcus spp. to methicillin is the utilization of heavy metals (1, 3, 4, 11-13). Although heavy metals have many uses in our lives, such as industrial uses, they are toxic, and long-term exposure to them may make bacteria resistant to these metals (14). Researchers have proved that in bacteria; resistance to antibiotics, is strongly and positively correlated with resistance to heavy metals (zinc and cadmium) (3). The simultaneous presence of both antibiotics and heavy metals in the bacterial environment results in co-resistance in bacteria. It is believed that during environmental co-contamination; heavy metals induce antibiotic resistance via the co-localization of genes involved in resistance to heavy metals and antibiotics (1, 4). Co-localization of *czrC* (gene of resistance to zinc and cadmium) and *mecA* (gene of resistance to methicillin) on the SCCmec element is a prominent example of this phenomenon (1, 4, 11). The exposure of bacteria to zinc occurs through the utilization of this element as an alimentary supplement or curative agent in animal nourishment, which in turn significantly results in resistance to antibiotics (1, 4). Studies in other countries show that the frequency rate of resistance to zinc chloride in staphylococci is variable, so zinc resistance (MIC>2mM) was observed in 74% and 42% of European MRSA CC398 from pigs and veal calves respectively, and in 44% of the Canadian isolates, but not among the Chinese isolates (12, 15, 16).

Cadmium pollutes the environment through agricultural, pharmaceutical, fertilizers, domestic sewage, atmospheric sources, and industrial activities (17). The aim of this study was to investigate the occurrence of the emerging antibiotic/ heavy metal-resistant bacteria, for the first time in Iran. In this study, by identifying MRS strains isolated from humans and domestic animals that were also resistant to zinc chloride and cadmium acetate, the correlation between resistance to these heavy metals and antibiotic resistance was examined. In addition, by evaluating the presence of genes *czrC* and *mecA* in staphylococcus spp. isolates, the correlation between these two genes was also examined.

Materials and Methods

Gathering of human and animal Staphylococcal isolates

In the present study, in the first step, human and domestic animal staphylococcal isolates were prepared from the collection of microorganisms in the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad in Khorasan province, northeast of Iran and the Faculty of Veterinary Medicine of Tehran University in Iran. The isolates had been previously obtained from bovine mastitis milk, and nasal swabs from healthy humans and healthy domestic animals (horses, dogs, and cats) from 2017 to 2021. The glycerinated stocks were cultured on sheep blood agar (Merck, Germany) at 37 °C and incubated for 48h. A single colony was picked and streaked on mannitol salt agar to check the purity of the colony. Then, morphological characteristics of the bacteria were examined by the Gram-staining and were subjected to catalase and oxidase tests (Microdase modifications) (3, 8).

Antibiotic susceptibility testing

After collecting 300 isolates of staphylococci from humans (n = 70), cows (n = 100), horses (n = 50), dogs (n = 50), and cats (n = 30), in each isolate, a confirmed colony, sub-cultured on brain heart infusion (BHI) agar (Merck, Germany) for 24 hours and then on MHA (Merck, Germany) for 24 hours to evaluate the phenotypic resistance to methicillin. In this research, antibacterial resistance to the two antibiotics, penicillin (10 µg) and cefoxitin (30 µg) was specified by disk diffusion method using MHA (Merck, Germany) for all bacterial isolates, which were identified as staphylococcus spp. (7, 18).

According to the CLSI guideline, the cefoxitin-disc diffusion method was used to evaluate methicillin resistance in all staphylococcus spp. isolates (7, 20). Following measuring the diameter of growth inhibition zones, the comparison of values was performed to detect MRS isolates using the CLSI standards 2018 (19, 20). After detecting MRS isolates, these colonies were sub-cultured on brain heart infusion (BHI) broth (Merck, Germany) for 24 hours and then were preserved at -20 ^{o}C by adding 25% sterile glycerol (3, 11).

Molecular detection of nucA, mecA, and czrC

For DNA extraction, the boiling method was utilized. From staphylococcal colonies of a 24-h culture on BHIA media, a filled ring was suspended in 400 µL of sterile distilled water in a 1.5 mL Eppendorf tube, vortexed, heated up to 100 °C for 15 min and placed in -20 °C for 15 min. Then it was centrifuged at a speed of 8,000 rpm for 5 min and finally 100 µL of supernatant was transferred to a new 0.5 mL Eppendorf tube (0.5 mL microtube) and used immediately (2, 11). Following DNA extraction of phenotypic MRS isolates, DNA templates were utilized in simplex polymerase chain reaction (PCR) test and observed by electrophoresis technique to distinguish the existence or lack of *nucA*, *mecA*, and *czrC* genes. The amplification of the *nucA* gene was used for molecular identification of S. aureus isolates (2, 11), and the presence of *mecA* and *czrC* genes was examined to evaluate genotypic resistance to methicillin and heavy metals (zinc and cadmium) respectively (11, 16). The primers and PCR program of this reaction are shown in Table 1.

Zinc chloride and cadmium acetate susceptibility testing

Among 300 staphylococcus spp. isolates, MRS isolates were phenotypically tested to evaluate the resistance to heavy metals using Muller-Hinton agar dilution (12, 13, 16). For assessment of heavy metal susceptibility, Minimum inhibitory concentrations (MICs) were determined for cadmium acetate and zinc chloride on Mueller-Hinton agar plates with two-fold dilutions of either cadmium (0.125 to 16 mM) or zinc (0.25 to 16 mM) with an adjusted pH of 5.5 and 7.4 for zinc chloride and cadmium acetate respectively (3, 4, 12, 16). The cutoff value to determine resistance was considered the MIC value of ≥4mM (4, 12, 16).

Statistical analysis

In this study, the IBM SPSS, version 26 was used to measure the relationships between data. Additionally, the X^2 test (Fischer's exact test) was used to specify the relationships between the discontinuous variables and after calculating the *p*-values, the value less than 0.05 was statistically considered as significant. The relationship between phenotypic resistance to methicillin and *mecA* as antibiotic resistance gene as well as between zinc and cadmium resistance and *czrC* as heavy metal resistance gene were appraised using Pearson's correlation test.

Results

Antibiotic susceptibility testing

The antibiotic resistance profile of staphylococcus species isolates is shown in Table 2. It was found that overall, 177 (59%) of the isolates were resistant to penicillin (10 µg), while 51 (17%) isolates were resistant to cefoxitin (30 µg) that were identified as MRS. All samples that were resistant to cefoxitin were also resistant to penicillin, but some isolates resistant to penicillin were susceptible to cefoxitin. In detail, 13 (18.6%), 27 (27%), 6 (12%), 3 (6%), and 2 (6.7%) of MRS isolates from humans, cattle, horses, dogs, and cats respectively (Table 2). The

frequency of MRS isolates among staphylococci isolated from humans and domestic animals is shown in Figure 1.

Molecular identification of S. aureus

All MRS isolates from humans and domestic animals were screened to identify the S. aureus strains using the PCR method for the presence of a specific *nucA* gene. However, 25 (49%) of MRS isolates were confirmed to be MRSA. In detail, 10 (77%) in humans, 10 (37%) in cattle, 4 (66.7%) in horses, 1 (33.3%) in dogs, and 0 (0%) in cat samples were MRSA isolates (Table 3). PCR amplification of the *nucA* gene for S. aureus is shown in Figure 2. Unlike cow and dog MRS isolates, most human and horse MRS isolates are MRSA.

Heavy metal susceptibility testing

Among 51 MRS isolates obtained from humans and domestic animals, 49% (n = 25) of isolates were phenotypically resistant to zinc chloride, while 8% (n = 4) were phenotypically resistant to cadmium acetate. Heavy metal susceptibility testing revealed that 74% of cow MRS isolates, 50% of horse MRS isolates and 66.7% of dog MRS isolates were resistant to zinc chloride. Whereas all MRS isolates in humans and cats were susceptible to zinc chloride. Overall, 66.7% of dog MRS isolates and 7.4% of cow MRS isolates were resistant to cadmium acetate but MRS isolates obtained from other groups were susceptible to cadmium acetate. The heavy metal resistance profiles of staphylococcus isolates in humans and domestic animals (cattle, horses, dogs, and cats) are shown separately in Table 4 and Figure 3. As shown in Table 5, the resistance of MRSA strain to heavy metals is greater than other MRS species. In cows, horses, and dogs, most of the MRS isolates that are resistant to heavy metals are MRSA.

Molecular detection of mecA and czrC genes

The *mecA*-specific PCR demonstrated that 25 of 51 (49%) of total *MRS* strains were positive for the presence of the *mecA* gene. In detail, 46.2%, 37%, 66.7%, 100%, and 100% of *MRS* isolates respectively in humans, cows, horses, dogs, and cats were positive for the presence of the *mecA* gene (Table 3). PCR amplification of *mecA* gene for staphylococcus spp. isolates is shown in Figure 4. These results and *p-values* indicate that phenotypic resistance to methicillin had a significant relationship and a high correlation with the existence of the *mecA* gene, especially in dogs, cats, and horses (Figure 5). The *p*-values in humans, cattle, horses, dogs, and cats were less than 0.05 ($p < 0.05$). As shown in Table 6, the majority of MRS isolates that contain the *mecA* gene are MRSA, and no other staphylococcus spp. Most of the staphylococci containing *mecA* are MRSA

PCR of the c*zrC* gene showed that all isolates obtained from cattle and horses that were phenotypically resistant to zinc chloride were negative for the presence of the *czrC* gene but 66.7% of dog isolates that were phenotypically resistant to heavy metals had the *czrC* gene (Table 4).

PCR amplification of *czrC* gene for staphylococcus spp. isolates is shown in Figure 6. These results indicate that all staphylococcus isolates in humans and cats neither were resistant to heavy metals nor had the *czrC* gene. But some staphylococcus spp. isolates in cattle and horses that are phenotypically resistant to heavy metals lack the *czrC* gene, so phenotypic resistance to heavy metals does not correlate with the existence of the *czrC* gene. The results in dog MRS isolates indicate that phenotypic resistance to heavy metals has a significant relationship with the existence of the *czrC* gene (Table 4), and there is a high correlation between *mecA* and *czrC* genes. In dog isolates, the *p*-value is 0.001 and Pearson's correlation is 0.8 (Tables 3 and 4).

Table 1- PCR program and nucleotide sequences of primers utilized for amplification of specific genes involved in resistance to antibiotics and heavy metals in our research.

Target gene	Primer nucleotide sequence	Size of amplicon (bp)	Reference
nucA	F: 5´GCGATTGATGGTGATACGGTT3´ R:5´AGCCAAGCCTTGACGAACTAAAGC3´	270	11
mecA	F: 5´AAAATCGATGGTAAAGGTTGGC3´ R: 5´AGTTCTGCAGTACCGGATTTGC3´	533	2
czrC	F: 5´TAGCCACGATCATAGTCATG3´ R: 5´ATCCTTGTTTTCCTTAGTGACTT3´	660	16

Table 2. Frequency of MRS isolates among Staphylococci obtained from humans and domestic animals in this study

	Human (n = 70)	Cow (n =100)	Horse (n =50)	Dog (n = 50)	Cat (n = 30)	Total (n = 300)
Cefoxitin-resistant Staphylococci (MRS)	13 (18.6%)	27 (27%)	6 (12%)	3 (6%)	2 (6.7%)	51 (17%)
Penicillin-resistant Staphylococci	45 (64%)	71 (71%)	26 (52%)	17 (34%)	18 (60%)	177 (59%)

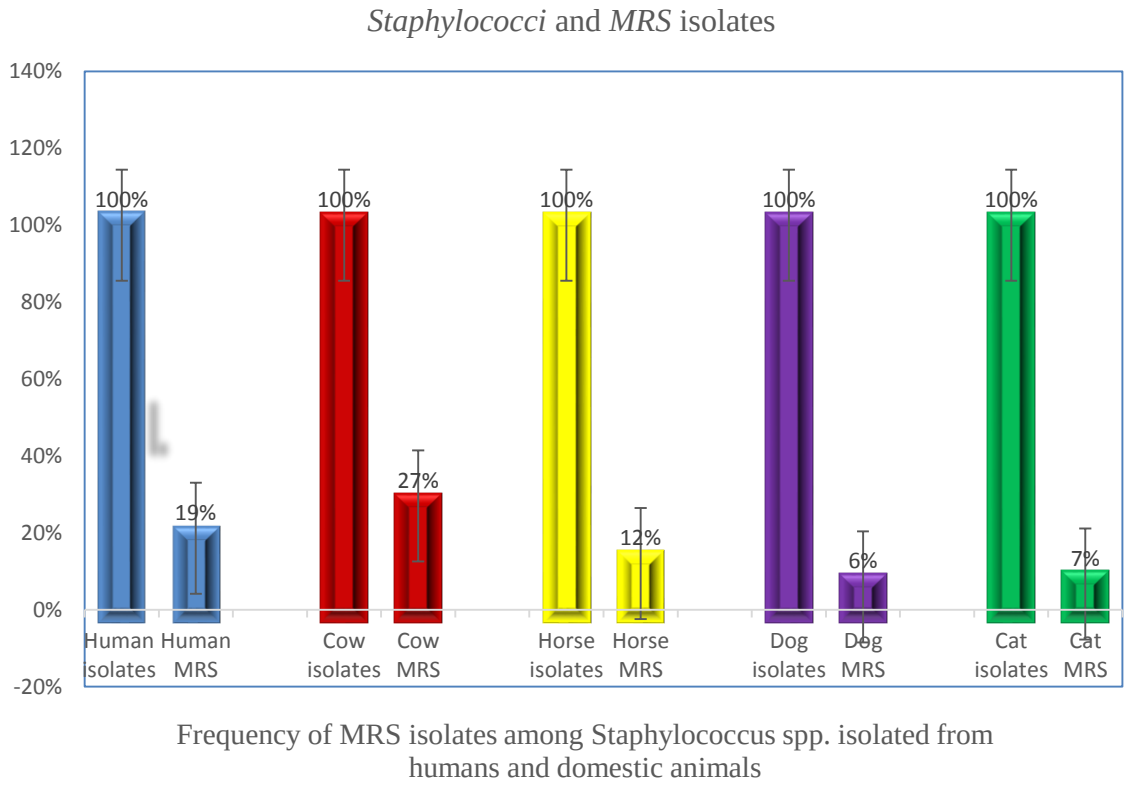

Fig. 1. Frequency of MRS isolates among staphylococi isolated from humans and domestic animals

Table 3. Prevalence of *nucA* and *mecA* in MRS isolates and frequency of MRSA and Other MRS strains.

	Human (n = 13)	Cow (n = 27)	Horse (n = 6)	Dog (n = 3)	Cat (n = 2	Total (n = 51)
MRSA- *nucA* gene-positive	10 (77%)	10 (37%)	4 (66.7%)	1 (33.3%)	0 (0%)	25 (49%)
Other MRS strains	3 (23%)	17 (63%)	2 (33.3%)	2 (66.7%)	2 (100%)	26 (51%)
mecA -PCR Prevalence	6* (46.2%)	10* (37%)	4* (66.7%)	3* (100%)	2* (100%)	25* (49%)

* Statistically significant ($p < 0.05$)

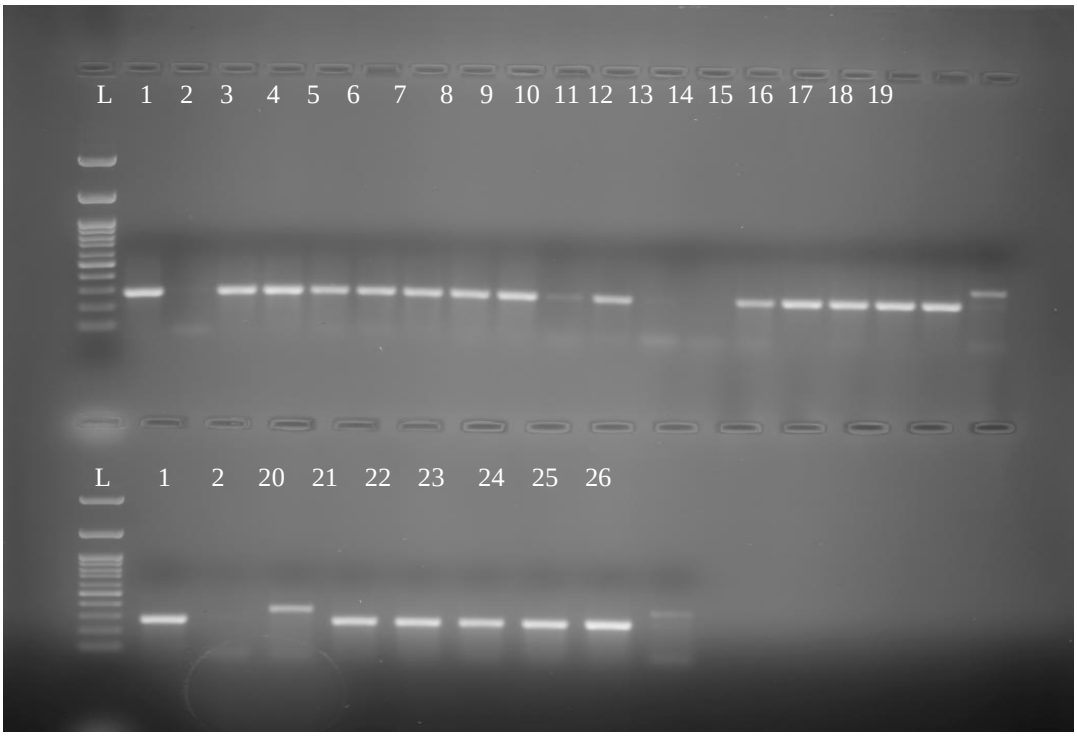

Fig. 2. PCR amplification of *nucA* gene for *S. aureus*. Lane L: DNA marker; 100 bp plus. Lane 1: Positive control. Lane 2: Negative control. Lane 3-26: test isolates. The expected product size of *nucA* is 270 bp.

Table 4. Frequency and number of MRS isolates resistant to heavy metals and prevalence of czrC gene in MRS isolates in this study

	Human (n = 13)	Cow (n = 27)	Horse (n = 6)	Dog (n = 3)	Cat (n = 2)	Total (n = 51)
MRS isolates resistant to Zinc chloride	0 (0%)	20* (74%)	3* (50%)	2* (66.7%)	0 (0%)	25 (49%)
MRS isolates resistant to Cadmium acetate	0 (0%)	2 (7.4%)	0 (0%)	2* (66.7%)	0 (0%)	4 (8%)
czrC PCR Prevalence	0 (0%)	0 (0%)	0 (0%)	2* (66.7%)	0 (0%)	2 (4%)

* Statistically significant ($p < 0.05$)

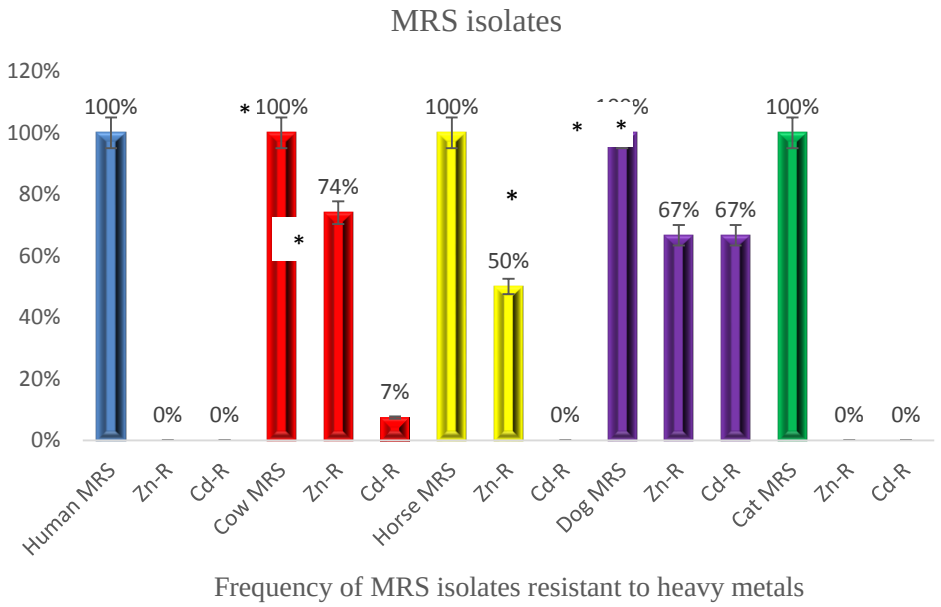

Fig. 3. Frequency of MRS isolates resistant to heavy metals among MRS isolates

Table 5. Comparison of the frequency of MRSA isolates and other MRS (other than MRSA) isolates resistant to zinc chloride and Cadmium acetate in this study

	Resistant to zinc chloride	**Resistant to Cadmium acetate**
Human *MRSA* (n =10)	0% (n = 0)	0% (n = 0)
Human other *MRS* (n =3)	0% (n = 0)	0% (n = 0)
Cow *MRSA* (n =10)	90% (n = 9)	10% (n = 1)
Cow other *MRS* (n =17)	65% (n = 11)	6% (n = 1)
Horse *MRSA* (n = 4)	75% (n = 3)	0% (n = 0)
Horse other *MRS* (n = 2)	0% (n = 0)	0% (n = 0)
Dog *MRSA* (n = 1)	100% (n = 1)	100% (n =1)
Dog other *MRS* (n = 2)	50% (n = 1)	50% (n =1)
Cat *MRSA* (n = 0)	0% (n = 0)	0% (n = 0)
Cat other *MRS* (n = 2)	0% (n = 0)	0% (n = 0)
Total *MRSA* (n = 25)	52% (n =13)	8% (n = 2)
Total other *MRS* (n = 26)	46% (n = 12)	7.7% (n = 2)

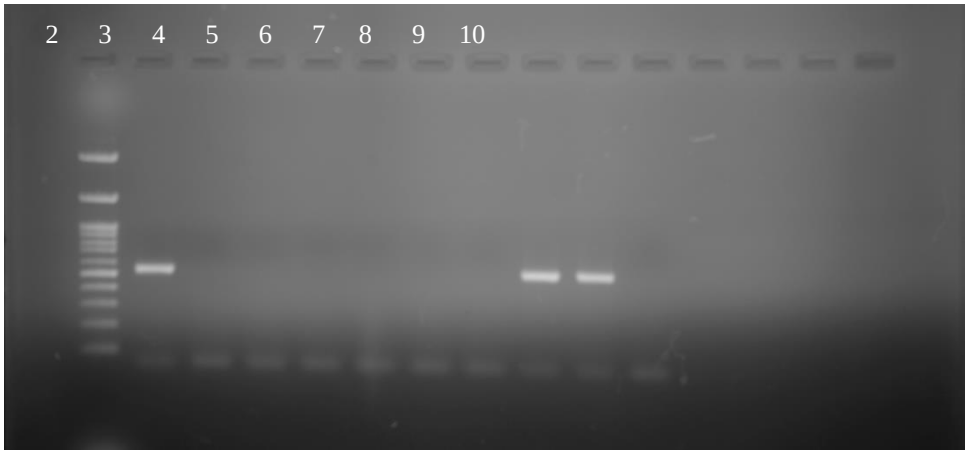

Fig. 4. PCR amplification of *mecA* gene for Staphylococcus spp. isolates. Lane L: DNA marker; 100 bp plus. Lane 1: Positive control. Lane 2: Negative control. Lane 3-10: test isolates. The expected product size of *mecA* is 533 bp.

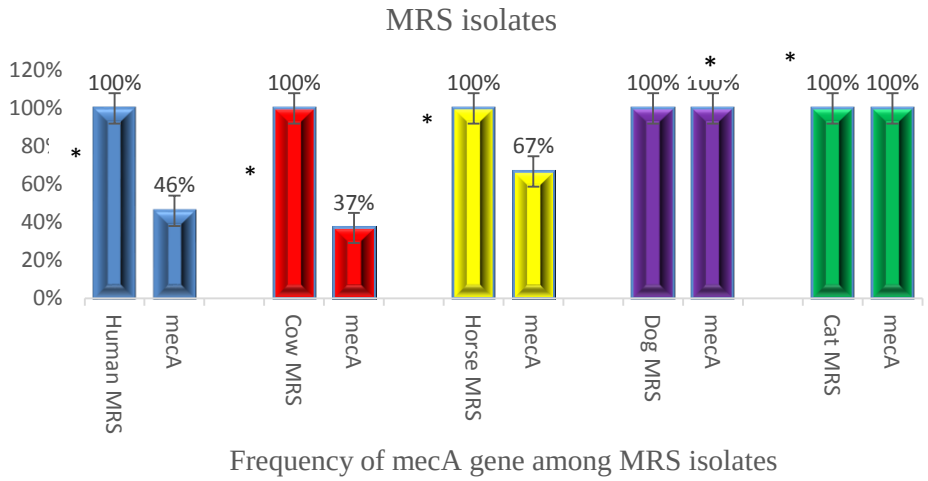

Fig. 5. Frequency of *mecA* gene among MRS isolates

Table 6. Frequency of MRSA and other MRS isolates among MRS isolates containing *mecA*

	Human (n = 6)	Cow (n = 10)	Horse (n = 4)	Dog (n = 3)	Cat (n = 2)	Total (n = 25)
Other MRS	0 (0%)	4 (40%)	0 (0%)	2 (66.6%)	2 (100%)	8 (32%)
MRSA	6 (100%)	6 (60%)	4 (100%)	1 (33.3%)	0 (0%)	17 (68%)

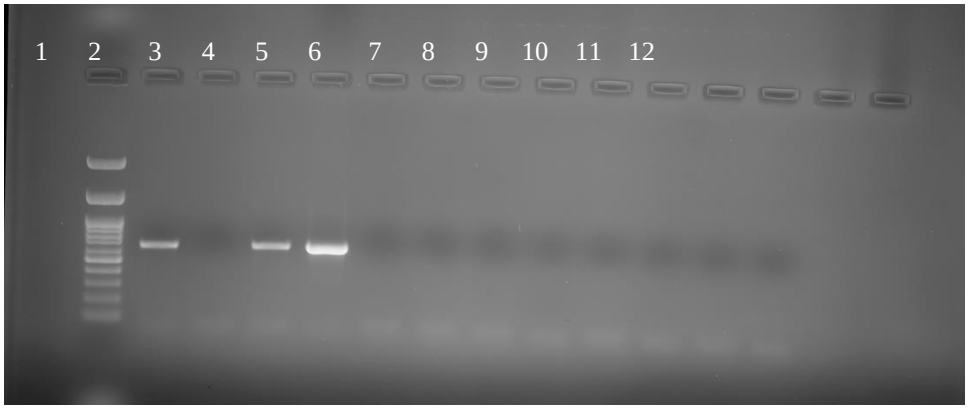

Fig. 6. PCR amplification of *czrC* gene for Staphylococcus spp. isolates. Lane L: DNA marker; 100 bp plus. Lane 1: Positive control. Lane 2: Negative control. Lane 3-12: test isolates. The expected product size of *czrC* is 560 bp.

Discussion

Staphylococcus is an important pathogen with a zoonotic potential that causes a variety of diseases (11). The WHO has reported that the treatment of many infections is difficult or sometimes impossible and this is due to the irregular utilization and injudicious consumption of antibiotics (11, 21). Similar to bacterial contact with antibiotics, the contact of bacteria with heavy metals leads to resistance (11, 20). The co-existence of antibiotic and heavy metal-resistance genes, such as *mecA* and *czrC* in the staphylococcal cassette chromosome (*SCC*) element results in resistance to antibiotics via the co-selection phenomenon (11, 22, 23). In this study, as shown in Table 3, there was a significant relationship between phenotypic resistance to methicillin and the existence of *mecA* gene in all MRS strains of human and domestic animals especially in MRSA isolates.

Several studies have shown that some staphylococcus spp. isolates that lack the *mecA* gene are phenotypically resistant to methicillin (9, 11, 24, 25). Similarly, in this research, *mecA* was not identified in some examined staphylococcus spp. isolates in humans, cows, and horses and this may be a consequence of the presence of other genes such as *mecC* that have not been investigated in this research (2, 10, 11, 26-29). Studies have demonstrated that livestock animals may be reservoirs of *mecC*-methicillin resistant staphylococcus spp. (11, 26, 30-32). Although systems such as *mecA-mecI-mecRI* and *blaZ-blaI-blaR1*, which produce PBP2a, are the reasons for the emergence of methicillin-resistant strains, however, *femA* gene expression is essential for the expression of genes involved in methicillin resistance (11, 33, 34). In this study, phenotypic resistance to heavy metals (zinc and cadmium salts) as well as the presence and prevalence rate of *czrC* gene that is involved in genotypic resistance in staphylococcus spp. were also studied for the first time in Iran.

In this research, unlike the research done in other countries, in addition to the resistance of MRSA against heavy metals (zinc and cadmium), the resistance of other MRS isolates was investigated. Moreover, the studied isolates were humans and domestic animals (cows, horses, dogs, and cats), not pigs. In some studies, conducted in other countries, a strong correlation was reported in particular clonal lineages like LA-MRSA ST398 (4, 12, 15). The relatedness of this mechanism originates from Danish pigs that extensive utilization of diets containing zinc in weaned pigs as a substitute for antibiotics in the prevention or treatment of enteric infections pursued the forbidding of antibiotics for maturation advancement in 2000, nearly a decennium before LA-MRSA *ST398* became greatly widespread in these pigs (4, 12, 16, 18). There is a documentary relation between the existence of *czrC* and *mecA* genes (4, 15). The physical connection of the *mecA* gene with the *czrC*

gene is of special prominence because it perpetuates methicillin resistance through the phenomenon of co-selection when using zinc-containing diets in pigs (4, 13, 15). Like studies in other countries, in this investigation, as shown in Table 4, the prevalence of *czrC* gene was high in dog MRS isolates (66.7%), but it was not present in other (human, cow, horse, and cat) isolates. Based on the chi-square and two-sided Pearson's correlation tests, there was a significant relationship ($p = 0.001$) between phenotypic resistance to heavy metals (zinc and cadmium) and the existence of *czrC* gene in dog MRS strains. Meanwhile, the *mecA* gene had a high correlation (C = 0.8) with *czrC* gene in dog MRS strains (Tables 3 and 4).

But on the other hand, in human, cow, horse, and cat MRS strains, there wasn't any significant relationship between phenotypic resistance to heavy metals (zinc and cadmium) and the existence of *czrC* gene and there wasn't any correlation between the *mecA* and *czrC* genes. Despite the absence of the *czrC* gene, phenotypic resistance to zinc chloride has been observed and this could be due to another mechanism for the resistance against zinc (4, 12, 15). It is believed that negative isolates for the *czrC* gene may contain a kind IVa instead of the *SCCmec* element.

In future studies, isolates that are phenotypically resistant to zinc and cadmium and lack the *czrC* gene should be screened for other genetic mechanisms of zinc and cadmium resistance. However, because of the presence of *czrC* gene, pathogenic bacteria are also a major threat to public health due to their mechanisms of resistance to antibiotics and heavy metals. Although it was assumed that farm animals could harbor heavy metal resistance genes, in the present work they were only detected in dog MRS. It is of particular interest as companion animals are in close contact to humans. The underlying reasons need further research in the future. Generally, heavy metals should therefore be rationally used as therapeutic agents in animal feed, and heavy metal contamination and the use of antibiotics should be managed in the environment, for example, phosphorus fertilizers should be used rationally in agriculture.

Abbreviations

MRS*:* Methicillin-resistant *Staphylococci*

MRSA: methicillin-resistant *Staphylococcus aureus*

PCR: Polymerase chain reaction

PBP2a: Penicillin binding protein2a

S. aureus: Staphylococcus aureus

S. intermedius: Staphylococcus intermedius

MHA :Mueller Hinton agar

BHI: Brain heart infusion

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Conflict of interests

There are no conflicts of interest.

Ethical approval

All stages of the present work has been conducted according to the research ethics guidelines of Ferdowsi University of Mashhad and Ministry of Science.

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