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Molecular Typing of *Staphylococcus aureus* Isolated from Canine Pyometra

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ABSTRACT

Background: Pyometra is a chronic disease condition in bitches, characterised by the accumulation of pus within the uterus. Multi-drug resistant bacterial strains with biofilm-forming ability are one of the most significant reasons behind the failure of medical management. The present study was envisaged for the detection of antibiotic resistance, biofilm-forming ability and molecular typing of *S. aureus* isolated from canine pyometra.

Methods: A total of 40 samples were collected from dogs with pyometra and following isolation and identification, DNA was extracted from the pure cultures of 13 coagulase positive *Staphylococcus* isolates.

Result: Six isolates turned out to be *Staphylococcus aureus* in *nuc* gene specific PCR and in antibiogram, all were found to be multidrug resistant. Out of the 13 staphylococcal isolates, eight turned out be positive for the presence of *mecA* gene, which included five *S. aureus* isolates. Out of 13 isolates, 53.8% were found to be strong biofilm producers in tissue culture plate method. Among the 13 staphylococcal isolates, 53.8% showed the presence of *icaD* gene, whereas, 46.1% showed the presence of *bap* gene. All the six *S.aureus* isolates obtained in the study were subjected to *agr* typing by PCR and all belong to *agr* type I. All the isolates turned out to be belonging to *spa* type t037.

Key words: Antimicrobial resistance, Biofilm, Multi-drug resistant, Pyometra, Tissue culture plate method.

INTRODUCTION

Canine pyometra is a serious and life-threatening inflammatory condition, characterised by the accumulation of pus in the uterus. Although *Staphylococcus* spp. and *Escherichia coli* are associated with the majority of the infections, *Klebsiella* spp., *Streptococcus* spp. and *Pseudomonas* spp. are also linked to the illness (Hagman, 2018; Chitra, 2024). In case of valuable breeding bitches, medical management is most often resorted, with a long duration of antibiotic treatment, but recurrences are common. The significant reason behind this is the problem of antibiotic resistance. Among the various reasons implicated for this resistance, the ability of the bacterial organism to build biofilm is highly relevant (Robaj *et al.,* 2016; Amrutha *et al.,* 2022).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a group of *S. aureus* that are genetically distinct from other strains and is responsible for several difficult-to-treat infections in humans. People who live near to animals, especially companion animals, may be more easily exposed to MRSA due to their proximity. Resistance to methicillin is mediated by the *mecA* gene, which encodes an altered penicillin-binding protein (PBP2a/ PBP22) and is carried on the staphylococcal cassette chromosome *mec* element (SCC*mec*) (Goudarzi *et al*., 2021). The biofilm-forming abilities have been thoroughly demonstrated in members of the genus *Staphylococcus*, such as *S. epidermidis* and *S. aureus* (Atshan *et al.,* 2012). Among the various proteins associated with biofilm formation in *S. aureus*, apart from acting as virulence factors, proteins coded by *ica*A, *ica*D and *bap* genes plays a significant role (Salina *et al.,* 2020). Pathogenic bacteria survive in different types of reservoirs, *viz*., humans, animals, water and food. Dissemination from these reservoirs produce clusters of colonization or infection, which is termed as outbreaks. In such conditions, typing is employed, either for surveillance of infectious diseases or in outbreak investigations.

To establish and maintain infection, *S. aureus* encodes several virulence factors and accessory gene regulator (*agr*) system is one among them, which regulates and controls the production of these virulence factors. Many genes involved in tissue colonisation and invasion are controlled by this system. *agr* I, *agr* II, *agr* III and *agr* IV are the four kinds of *agr* polymorphism that originate from mutation and/or insertions in *agr* C and *agr* D genes (Derakhshan *et al.,* 2021). Spa typing has been extensively

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used as a typing tool for epidemiological investigations and some researches recommend it for outbreak investigations and detection of source of transmission (Strommenger *et al.,* 2008). The typing relies on the assessment of number and sequence variation in repeats at the X region of the *spa* gene and it exhibits good discriminatory power and is very useful as it is easy to perform, cheap and have a standardized system of nomenclature (O Hara *et al*.,2016).

So far, in Kerala no studies have been conducted in this regard on canine pyometra caused by *S. aureus*. Hence, the present study is envisaged in this direction. The study will throw light on the association between the 3 different molecular types of *S. aureus*, its biofilm forming potential and resistance profile.

MATERIALS AND METHODS

A total of 40 samples were collected from bitches with pyometra. The samples included guarded anterior vaginal swabs from cases of open pyometra and were collected from Kerala Veterinary and Animal Science University hospitals at Mannuthy and Kokkalai during the year 2023. The samples were processed for bacterial isolation and identification employing standard microbiological procedures and the isolates were identified based on cultural, morphological and biochemical characteristics. *Staphylococcus aureus* DNA was extracted from one single colony employing commercial DNA extraction kit (HiPurA® Bacterial Genomic DNA Purification kit) according to the manufacturer's instructions. Isolated DNA served as the template for the detection of the *nuc gene*. Primers using forward (5′ -GCGATTGATGGTGATACGGTT-3′) and reverse (5′-AGCCAAGCCTTGACGAACTAAAGC-3′) sequences were used to amplify the *S. aureus nuc* gene.

The PCR program consisted of an initial denaturation step at 95°C for 5 min, denaturation in 35 Cycles of 60 s at 94°C, annealing at 55°C for 30 s and extension at 72°C for 90 s; and final extension in 1 cycle of 72°C for 10 min. The expected amplicon size was 270 bp.

Antibiogram

Kirby-Bauer disc diffusion method was performed on MHA plates to determine the susceptibilities of the isolates to different beta-lactam and non-beta-lactam antibiotics (CLSI, 2018).

Multiple antibiotic resistance index (MAR) of the isolates was calculated as the ratio of number of antibiotics to which organism is resistant to total number of antibiotics to which organism is exposed. The MAR index value greater than 0.2 indicate high risk source of contamination, where the antibiotics are often used. The susceptibility test of the isolates was done on MHA by Kirby Bauer disc diffusion method. Antibiotics used were cefoxitin and oxacillin. The zones of inhibition were measured and the results interpreted according to the guidelines of Clinical Laboratory Standard Institute (CLSI, 2018). *Staphylococcus aureus* DNA was extracted employing commercial DNA extraction kit. Amplification of *mecA* and *mecC* were performed using forward primer *mecA*-FP-AAAATC GATGGTAAAGGTTGGC and reverse primer *mecA*-RP- AGT TCTGC AGTACCGGATTTGC and *mecC*-FP- GAAAAAA AGGCTTAGAACGCCTC and reverse primer sequence *mecC*-RP-GAAGATCTTTTCCGTTTTCAGC (Goudarzi *et al*., 2021). PCR was performed as per the protocol described with minor modification with the following thermal cycling conditions: Initial denaturation at 95°C for 5 min followed by 35 cycles of amplification at 95°C for 30 s, 52°C for 60 s, 72°C for 60 s and final extension at 72°C for 10 min. PCR amplified a 533 bp fragment of the *mec A* and 138 bp fragment of *mecC* gene.

Detection of biofilm

Biofilm forming potential of the *S.aureus* isolates was determined quantitatively by tissue culture plate method, quantitative method was described by Christensen *et al*. (1985). Organism isolated from fresh agar plates were inoculated in 10 mL TSB with 1 per cent glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted to 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture plates were filled with 200 µL of the diluted cultures. The positive control *(E.coli*) and negative control (fresh TSB with 1 per cent glucose) were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24 h. Contents of each well was removed by gentle tapping. The wells were washed with 0.2 M of phosphate buffer saline (pH 7.2) four times. Biofilm formed by bacteria adherent to the wells were fixed using 2 per cent sodium acetate and stained with 0.1 per cent crystal violet. Excess stain was removed using deionized water. Crystal violet remaining in the wells was then dissolved in 1mL of an ethanol/acetone solution. Optical density (OD) of stained adherent biofilm was obtained using microELISA auto reader at a wavelength 570 nm. The isolates showing an average OD value more than 0.240 were considered as strong biofilm producers.

Detection of virulence genes

The presence of the virulence genes *ica*A, *ica*D and *bap* associated with *S. aureus* isolates were detected employing PCR. *ica*A F (5′ - CCTAACTAACGAAAGGTAG -3′), *ica*A R (5′-AAGATATAGCGATAAGTGC -3′), *ica*D F (5′-AAACGTAAG AGAGGTGG -3′) *ica*D R (5' - GGCAATATGATCAAGATAC -3′), *bap* F (5′- CCCTATATCGAAGGTGTAGAATTG -3′) and *bap* R (5′ - GCTGTTGAAGTTAATACTGTACCTGC - 3′). These primers allow the amplification of a 1315-bp DNA fragment for *ica*A, 381-bp DNA fragment for *ica*D and971-bp DNA fragment for *bap*. The primers used in the study for the detection of the virulence genes were selected as per Nemati *et al.* (2009). Amplifications were carried out in an MJ Mini Bio-Rad thermal cycler through the following temperature program for the amplification of *ica*A and *ica*D genes: 1 cycle of 5 min at 94°C; 30 cycles of 45 s at 94°C,

45 s at 55°C and 45 s at 72°C; and finally 1 cycle of 72°C for 10 min and for amplification of *bap* gene :1 cycle of 5 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 61.3°C and 45 s at 72°C; and finally 1 cycle of 72°C for 10 min. Amplification products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by transillumination under UV.

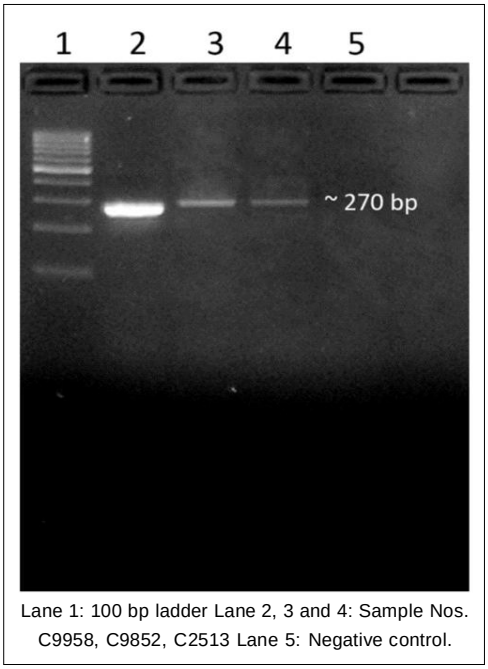
Molecular typing of *S. aureus*

The presence or absence of the genes *agr* I, *agr* II, *agr* III and *agr* IV in *S. aureus* isolates was determined by constructing a quadruplex PCR. The quadruplex PCR was performed in a total volume of 20 µL reaction mixture. Pan (5 -ATG CAC ATG GTG CAC ATG C-3), *agr* I (5 - GTC ACA AGT ACT ATA AGC TGC GAT-3), *agr* II (5 -TAT TAC TAA TTG AAA AGT GGC CAT AGC-3), *agr* III (5 -GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3) and *agr* IV(5 -CGA TAA TGC CGT AAT ACC CG-3) (Kahl *et al*., 2005; Goudarzi *et al*., 2019). These primers allow the amplification of a 441-bp DNA fragment for *agr* I, of a 575-bp fragment for *agr* II, a 323-bp fragment for *agr* III and a 659-bp fragment for *agr* IV strains. Amplifications were carried out in an MJ Mini Bio-Rad thermal cycler through the following temperature program: 1 cycle of 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C; and finally 1 cycle of 72°C for 10 mins. Primers *spa* F (5'- TAAAGACGATCCT TCGGTGAGC- 3') and *spa* R (5' - CAGCAGTAGTGCCGTTT GCTT - 3') were used to amplify a portion of *spa* gene (products variable 100 - 600 bp). The PCR program consisted of an initial denaturation step at 80°C for 5 min, denaturation in 35 cycles of 45 s at 94°C, annealing at 600C for 45 s and extension at 72°C for 90 s; and final extension in 1 cycle of 72°C for 10 min.

RESULTS AND DISCUSSION

A total of 40 samples, Gram's staining revealed that 17 isolates were Gram positive and 23 were Gram negative bacilli. The 13 samples were produced white or yellow large smooth colonies on BHIA with positive reaction for catalase, methyl red, VP, citrate utilisation, urease and coagulase test and negative results for indole production and oxidase test. All of them, which produced yellow coloured colonies on MSA and tube and slide test positive for coagulase, were confirmed as coagulase positive *Staphylococcus* spp. The *nuc* gene specific PCR for *S. aureus* was carried out with the DNA of 13 coagulase positive *Staphylococcus* isolates and 46.1% turned out to be positive for *S. aureus* (Fig 1). Brakstad *et al*. (1992), employed PCR targeting the region of the *nuc* gene, that specifically codes for the thermostable nuclease seen in *S. aureus*, for the specific identification of the species.

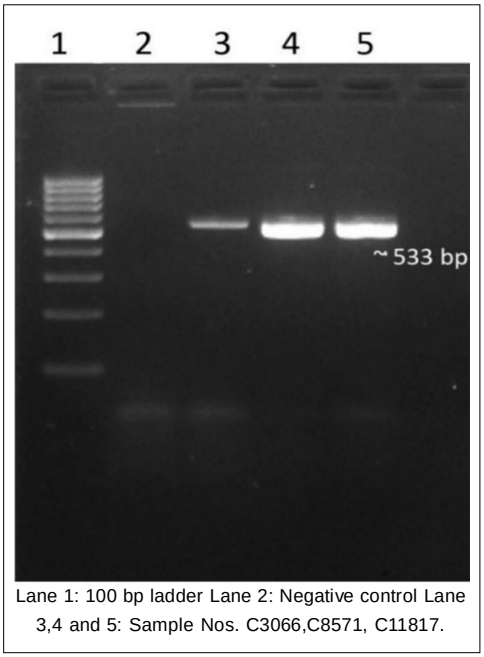
The 32.5% *Staphylococcus* spp. obtained in the study were subjected to antibiogram employing the 20 antibiotics, commonly employed for the therapy of infections in dog. All the isolates were found to be MDR, showing resistance to at least two classes of antibiotics. Similar findings were documented by Amrutha *et al*. (2022), who used the typical antibiotics used to treat pyometra to antibiogram the isolates. While 100% of S. aureus isolates were resistant to amoxy-clav, ceftriaxone, ceftriaxone-tazobactam, ciprofloxacin, gentamicin and metronidazole, 50% of them were sensitive to tetracycline, followed by enrofloxacin (37.5%) and co-trimoxazole (25%). Subramani and Vignesh (2012) conducted a study on the MAR index of the *S. aureus* isolates from clinical pus samples, using 17 distinct antibiotics, belonging to the class β-lactams, aminoglycosides and quinolones. The study recorded that 50 per cent of the isolates were MDR and every isolate had an extremely high MAR Index, indicating that the isolates were likely originated from areas with extensive antibiotic use. Phenotypic characterisation of the isolates employing oxacillin disc diffusion test, among the 13 *Staphylococcus* isolates, 69.2% showed resistance to methicillin on oxacillin disk diffusion test, which included 66.6% *S. aureus* and 33.3% other coagulase positive *Staphylococcus* spp and 30.7% coagulase positive *Staphylococcus* spp. All the 13 isolates were subjected to PCR targeting the *mec*A and *mec*C genes and 61.5% turned out be positive for the presence of *mec*A gene, which included five*S. aureus* isolates and three other coagulase positive *Staphylococcus* spp. 38.4% isolates did not show the presence of *mec*A gene (Fig 2). None of the isolates were positive for the presence of *mec*C gene. One coagulase positive *Staphylococcus* spp. which showed resistance phenotypically, gave negative results for the presence of *mec*A gene. Similar observations were documented by Abdelwahab *et al*. (2023) in a study on the phenotypic and genotypic characterisation of methicillin resistance



Lane 1: 100 bp ladder Lane 2, 3 and 4: Sample Nos. C9958, C9852, C2513 Lane 5: Negative control.

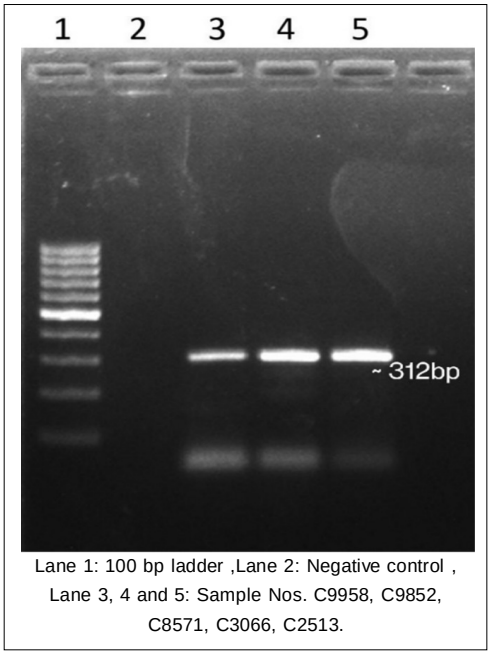
Fig 1: Agarose gel electrophoresed image of *nuc*gene of *Staphylococcus aureus.*

in *Staphylococci* isolated from an Egyptian University Hospital. If a *Staphylococcus* spp. is methicillin and oxacillin resistant phenotypically, but genotypically negative for *mecA* gene, it could be due to the presence of *mecC* gene, which is a homolog of *mecA* gene and is also responsible for methicillin resistance in some staphylococcal isolates. A study on MRSA screening in *S. aureus* by Becker *et al*. (2018), documented that a *mecB* homolog with 60% nucleotide sequence similarity to the originally identified *mecA* gene of *S. aureus could also be responsible for methicillin resistance in the isolates that tested negative for mecA and mecC. Also,* Ba *et al*. (2014), mentioned that specific



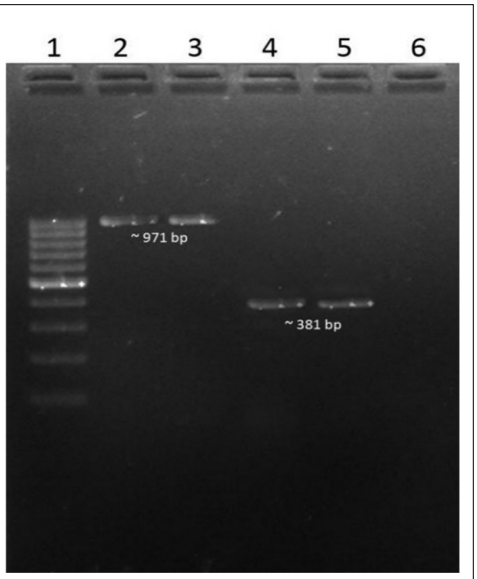
Lane 1: 100 bp ladder Lane 2: Negative control Lane 3,4 and 5: Sample Nos. C3066,C8571, C11817.

Fig 2: Agarose gel electrophoresed image of *mec*A gene of *Staphylococcus aureus.*



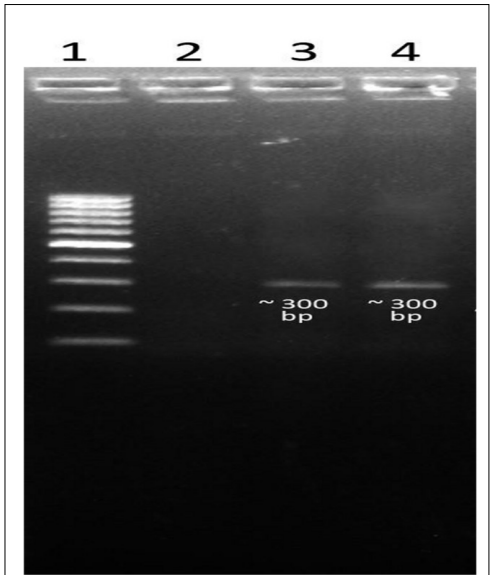
Lane 1: 100 bp ladder ,Lane 2: Negative control , Lane 3, 4 and 5: Sample Nos. C9958, C9852, C8571, C3066, C2513.

Fig 4: Agarose gel electrophoresed image of *agr*gene of *Staphylococcus aureus.*



Lane 1: 100 bp ladder Lane 2 and 3: Sample Nos. C3066, C8571 Lane 4and5: Sample Nos. C3066, C8571, Lane 6 : Negative control.

Fig 3: Agarose gel electrophoresed image of bap and icaD gene of *Staphylococcus aureus*.



Lane 1: 100 bp Ladder, Lane 2: Negative control, Lane 3 and 4: Sample Nos. C8571, C3066.

Fig 5: Agarose gel electrophoresed image of *spa* gene of *Staphylococcus aureus.*

alterations in different amino acids present in protein binding proteins cascade (PBPs 1, 2 and 3) might also be the basis of methicillin resistance.

Out of the 13 isolates, 53.8% were found to be strong biofilm producers in tissue culture plate method (Table 1). Mishra *et al*. (2015), conducted studies for comparing the different methods for detection of biofilm formation in clinical isolates of *S. aureus* from indwelling medical devices and out of 67 isolates, 31 (46.3 per cent) were found to be biofilm producers and 36 (53.7 per cent) were non-biofilm producers by tissue culture plate method. The study documented that tissue culture plate method is the gold standard method for the detection of biofilm producers and in addition, they recommended tissue adherence method for routine detection of biofilm production in countries, where molecular methods and sophisticated microscopy techniques are constrained. The presence of virulence genes associated with biofilm formation, *ica*A, *ica*D and *bap*among the *Staphylococcus* spp. were detected employing PCR. All the six *S.aureus* isolates and one coagulase positive *Staphylococcus* spp. showed the presence of *ica*D gene, whereas, five *S.aureus* and one coagulase positive *Staphylococcus* spp. showed the presence of *bap* gene (Fig 3). None of the isolates revealed the presence of *ica*A gene. Similar results were reported by Grinholc *et al*. (2007) in a study, who documented that 91 per cent of MRSA strains harbored *ica*D gene and in contrary, all *ica*D negative strains were*ica*A positive. Nourbakhsh *et al*. (2016) in a study on the phenotypic and genotypic characterization of biofilm formation in MRSA, documented that *ica*C gene had the highest frequency among them, followed by *ica*D.

All the six *S.aureus* isolates obtained in the study were subjected to *agr* typing by PCR followed by nucleotide sequencing. All the isolates in the present study were found to be belonged to *agr* type I (Fig 4).Bibalan *et al*. (2014), who evaluated the *agr* types of *S. aureus,* obtained from patients, health care workers and food products, to assess the possible relationship between agr groups and infection types and the study reported that majority of the isolates belonged to *agr* I, followed by *agr* III, II and IV.All the six *S.aureus* isolates obtained in the study were subjected to spa typing by PCR followed by nucleotide sequencing of the representative amplicons. The sequences were submitted to spa Typer online software (http://spatyper.fortinbras.us/). The results were then compared to an online website for world spa types (Ridom spa Server, https://www.spaserver.ridom.de/).The isolates turned out to be belonged to spa type t037 (Fig 5). Singh *et al.* (2018) conducted a study on molecular epidemiology of MRSA, which identified 29 MRSA isolates with 9 unique *spa* types, with t037 (n = 6) and t045 (n = 6) being predominant, which was in accordance with the results of the present study.

The linkage between antibiotic resistance shown by the *S. aureus* isolates, their biofilm forming potential and the molecular types to which they belonged was assessed. All the isolates were found to be MDR, five of them were MRSA and three were strong biofilm formers. All the isolates belonged to *agr* type I and *spa* type t037. Since the number of *S. aureus* isolates obtained in the study was less and all the isolates belonged to same *agr* type and *spa* type, the linkage between the antibiotic resistance, biofilm forming potential and molecular types of the isolates could not be deducted (Table 2). Asadollahi *et al*. (2018) conducted a study on available literatures to identify the most prevalent *spa* types among clinical isolates of MRSA and methicillin susceptible *S.aureus* around the world and documented that the most prevalent *spa* types identified in Asia were t037 and t002 and majority of the isolates under the type t037 were MRSA. Rofik *et al*. (2024) conducted a study on the characterisation of biofilm formation in uropathogenic

Table 1: Optical density values in tissue culture plate method for biofilm detection of *Staphylococcus* spp. at 590 nm.

Sample no	OD values		Average OD	
Positive control	0.379	0.365	0.171	0.303
Negative control	0.072	0.053	0.056	0.060
C8571: *S. aureus*	0.39	0.539	0.483	0.470
C3066: *S. aureus*	0.169	0.212	0.224	0.202
C10107: *S. aureus*	0.392	0.206	0.391	0.329
C9852: *S. aureus*	0.077	0.075	0.066	0.073
C9958: *S. aureus*	0.184	0.183	0.209	0.192
C2513: *S. aureus*	0.423	0.367	0.452	0.414
C10432	0.063	0.064	0.112	0.080
C7360	0.362	0.502	0.308	0.390
C9852	0.258	0.385	0.449	0.364
C11058	0.510	0.446	0.677	0.544
C11282	0.207	0.120	0.133	0.154
C5138	0.064	0.057	0.058	0.060

Table 2: Linkage between antibiotic resistance, biofilm forming potential and molecular types of *S. aureus* isolates.

Isolates	Antibiogram	Biofilm			*agr*types	*spa*types
		Strong	Moderate	Weak		
C8571	MDR	S	-	-	*agr*I	t037
C3066	MDR	-	M	-	*agr*I	t037
C10107	MDR	S	-	-	*agr*I	t037
C9852	MDR	-	-	W	*agr*I	t037
C9958	MDR	-	M	-	*agr*I	t037
C2513	MDR	S	-	-	*agr*I	t037

S. aureus and their association with antibiotic resistance and reported that 55 per cent were found to be MDR and MRSA. In addition, all the strains were biofilm producers and a significant correlation was observed between MDR and non-MDR strains in terms of biofilm production. A study on genotypic characterisation of *S. aureus* isolated from a burn centre using *agr, spa* and SCC*mec* typing methods was conducted by Abbasian *et al*. (2018). The study documented that among the MRSA isolates, 78.4 per cent were characterised as *agr* type I and *spa* types t030 and t037 constituted the first and second most predominant *spa* types found in 56.4 per cent and 25.6 per cent of isolates, respectively. Cafiso *et al*. (2007) in a study examined the relationship between the development of biofilm and the accessory gene regulator (*agr*) in 29 strains of *S. aureus* isolated. Compared to other *agr* types, *agr* type II strains exhibited much higher biofilm-forming abilities.

CONCLUSION

In the present study, all the *S. aureus* isolates obtained were found to be MDR and five of them were MRSA strains with biofilm-forming abilities, a significant factor behind the complications in the medical management of pyometra with recurrence. All the isolates were found to be *agr* type I and *spa* type t037. Since many researches have documented that many of the MRSA isolates from human belonged to *agr* type I and *spa* type t037 and considering the fact that companion animals live in close contact with the human environment, these strains are a major public health concern, causing life-threatening complications in the therapy of clinical infections in human and animals.

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Conflict of Interest

All authors declared that there is no conflict of interest.

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