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Isolation of methicillin resistant *Staphylococcus aureus* (MRSA) from oral cavity of dogs

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Abstract

Development and widespread use of a variety of new generation of antibiotics has given rise to some bacteria have become resistant to many currently used antimicrobials. Of particular concern are *Staphylococcus aureus* that have become resistant to beta-lactam antibiotics. A total of 120 oral swabs from apparently health and clinical diseased cases dogs of age ranged from 6 months to 2 years, weighed from 8 to 45 kg, were collected from hospitals and clinics in Cairo Governorate are subjected to bacteriological examination for isolation of *S. aureus*. Results revealed that *Staphylococcus aureus* was isolated from (71) samples in a prevalence rate of 59.2% mean while 49 samples were negative (40.8%). *Staphylococcus aureus* found to be resistant to Amoxicillin, Ampicillin, Clindamycin, Erythromycin, methicillin, Oxacillin, Penicillin and Vancomycin. Also susceptible to Ciprofloxacin, Doxycycline, Levofloxacin, Linezolid, Moxifloxacin, Rifampicin Tigecycline and Trimethoprim/ Sulfamethoxazole. PCR was used on this study including 8 isolates and proved to be S. aureus by coagulase test mean while the other 3 was coagulase –ve, in Addion to the result of sensitivity test using methicillin. Detection mecA, tst and blaZ genes as antibiotic resistance genes and spa, coa and nuc genes as virulence genes. The result revealed that mecA was detected in (6) isolates out of (8) tested, blaZ genes detected in all tested while tst gene detected in (2) isolates. Regarding virulence genes, spa genes were detected in (6) isolates while coagulase genes (coa) was detected in (5) isolates and nuc gene was detected in (3) isolates.

Keywords: *Antimicrobial resistance, dogs, MRSA, resistance genes and virulence genes*

Introduction

Pet animals play a role in transmission of zoonotic agents between humans and animals as a source of zoonotic infections (**Kruse 2004),** The number of owned dogs and cats was dramatically increased in the Egyptian society. Dogs and cats become an integral part of households, sharing human lifestyles **(Abdel-Moein and Samir, 2011)**.

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains one of the most virulent human pathogens and has also recently been recognized as such in the veterinary settings. Companion animals, including dogs, cats, horses, small exotic animals, wildlife animals, and livestock, may constitute a reservoir for MRSA transmission to humans and vice versa. The evolution, emergence and risk factors for MRSA transmission among colonized or infected animals **(Petinaki, 2015).**

Methicillin-resistant Staphylococci (MRS) are important pathogens in human and veterinary medicine and are often multidrug resistant, extremely limiting therapeutic options. MRS are recognized as one of the most important risks for human and animal Health **(Becker *et al.,* 2014 and Morris *et al.,* 2017).**

MRSA strains are not just resistant to methicillin, they're resistant to all the antibiotics in the same drug family as methicillin (the beta lactams), including many common drugs such as penicillins and cephalosporins. Some strains of

MRSA are also resistant to other families of antibiotics, which can make them extremely difficult to treat **(Scott, 2017).**

Antimicrobial multidrug-resistant microorganisms (MDRO) can be transmitted between companion animals and their human owners **(Kaspar *et al.*, 2018).**

Effective antimicrobial preparations, other than antibiotics, are important for the treatment of potentially fatal drug-resistant infections.Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of hospital-acquired and post- operative infections **(Haitham *et al.*, 2019)**.

Household pets, can be colonized or infected with a wide variety of bacteria pathogenic to animals and people. However, the close contact between household pets and people offers favorable conditions for transmission by direct contact (e.g. petting, licking or physical injures) or indirect through contamination of food and domestic environments. Indeed, frequent sharing of skin microbiota between people and their dogs has been shown, thus emphasizing the role of contact **(Song *et al.*, 2013).**

Dog-to-human transmission of MRSA pathogens could occur through dog bites or contamination of human food and water by bacteria in dog saliva. Direct mouth-to-mouth contact by dogs and children as they play is one of the likely sources of infection. The buccal cavity and saliva of such dogs are known to harbor many facultative anaerobes and obligate aerobes, some of which are potential human pathogens, these potential pathogen include *Staphylococcus aureus*, *Eeschericichia coli* and others **(Roberts *et al.*, 2000 and Songer and Post, 2004).**
Particular concern is strains of *Staphylococcus aureus* that have become resistant to beta-lactam antibiotics over the past few years **(Loffler and Macdougall, 2007).**

Methicillin resistance is due to the acquisition of the *mecA* gene, that encodes a new protein designated PBP2a, belonging to a family of enzymes necessary in building the bacterial cell wall. PBP2a has a very low affinity for β-lactam antibiotics and confers resistance to methicillin and the other beta-lactams (**Pantosti *et al.*, 2007).**

Methicillin resistant *Staphylococcus aureus* (MRSA), are becoming a significant source of morbidity in the health care setting. MRSA isolates have been shown to be responsible for osteomyelitis, pneumonia, skin infections, arthritis, endocarditis, gastroenteritis, abscesses, and in some cases, even necrotizing faciitis **(Lowy, 1998 and Mainous *et al.*, 2006).**

Therefore, this work aimed to detect the prevalence of *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from of dogs and identify its antibiotic susceptibility of the isolates as well as detect the virulence and antibiotic resistances genes of (MRSA) isolates.

Materials and Methods

A- Animals samples and bacterial cultures:

A total of 120 oral swabs were collected using sterile swabs of apparent healthy and diseased dogs, the age from 6 months to 2 years, weighed from 8 to 45 kg, at some hospitals and clinics in Cairo Governorate. Swabs were put in ice box and send to laboratory without delay for bacterial culture and identification of *S. aureus*.

B- Isolation and identification of *Staphylococcus aureus* (Quinn *et al.*, 2011).

Collected swabs were cultivated into nutrient broth tubes and incubated aerobically at 37°C for 18 h and then cultured onto sheep blood agar and mannitol salt agar. The inoculated plates were incubated aerobically for 24 hours at 37°C. Colonies were identified as mannitol fermenting (yellow) colonies on mannitol salt agar and β hemolytic on blood agar. The suspected colonies were picked up and tested for Gram's reaction. Colonies showed Gram positive cocci that arranged in irregular clusters were tested for catalase and coagulase.

Coagulase test:

A tube coagulase test was performed on pure β haemolytic colonies using lyophilized rabbit plasma. Identified colonies were confirmed

as *S. aureus* when reacted positive with commercial latex agglutination test, Staphaurex® (Remel, Lonex, Kans) isolation and identification of *S. aureus* were performed according to **Forbes *et al.* (2007)**.

C-Antibiotic sensitivity test:
Isolates cultivated into Mueller Hinton broth tubes and incubated aerobically at 37 °C for 18 h and then cultured onto Mueller Hinton agar plates and incubated as above for antimicrobial susceptibility testing, which was carried out by the standard disk diffusion method according to the Clinical Laboratory Standards Institute **(CLSI., 2017)**. The following antibiotic discs were used: Amoxicillin (AML 20 µg), Ampicillin (Amp,15µg;), Ciprofloxacin (CIP 5µg), Clindamycin (DA 10µg), Doxycyclin (D 30µg), Erythromycin (E 15µg), Levofloxacin (LEV 5µg), Linezolid (LZD 30µg), Methicellin (MET 5µg), Moxifloxacin (MXF 5µg), Oxacillin (OXA 1µg), Penicillin (P10 µg), Rifampicin (Rd 5µg), Tigecycline **(**TGC 15µg**),** Trimethoprim/ Sulfamethoxazole (SXT 25 µg), Vancomycin (VA 30µg). Antibacterial sensitivity in relation to zone of inhibition interpreted by the manufacturing company (Oxoid).

Molecular Detection (detection of antibiotic resistance and virulence genes of MRSA strain.

1- Extraction of DNA
DNA extraction from some was performed from the (8) MRSA strain using the QlAamp DNA Mini kit (Qiagen, Ge m mjm, GmbH) with modifications from the manufacturer's recommendations. Briefly, 2.00µl of the sample suspension was incubated With 10 pi of proteinase: K and 200 µl of lysis buffer at 560C for 10 min. After incubation, 2.00 µl of 100% ethanol was added to the lysate. The sample was then washed and Centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100µl of elution buffer provided in the kit.

2- Oligonuclewotide Primer
Primers use was supplied from Metbion (Germany) are listed in table (I).
PCR amplification **(kummr *et al*., 2009 and Mason *et al.*, 2001)** Primers were utilized in n 25µlreaction containing 12.5µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20 pmolconcentration, 4.5µl of water, and 6µl of DNA template.
The reaction was performed in an applied bios stem 2720 thermal cycler.

3- Analysis of the PCR Products.
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in Ix TBE buffer at room temperature using gradients of SV/cm. For gel analysis, 15 -ial of the products was loaded in each gel slot. A 100 bp and I00 DNA Ladders (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data was analyzed through computer software.

Table (1). Cycling conditions, primers sequences, target genes and amplicon size

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification(35cycle)			Final extension	Reference
				Secondary denaturation	Anneal-ing	Exten-sion		
mecA	F-GTAGAA ATG ACT GAA CGTCCG ATA A R-CCA ATT CCA CAT TGT TTC GGT CTA A	310	94° C 5 min	94° C 45 sec	94° C 45sec	72°C 45 sec	72°C 10 min	**McClure *et al*., (2006)**
blaZ	F- ACTTCAACAC-CTGCTGCTTTC R-TGACCAC-TTTTATCAGCAACC	173	94° C 5 min	94° C 30 sec.	54°C 30 sec.	72° C 30 sec.	72°C 7 min.	**Duran *et al*., (2012)**
tst	F-ACCCCTGTTCCCTTAT CATC R-TTTTCAGTATTT-GTAACGCC	326 bp	94˚C 10min.	94˚C 45sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Mehrotra *et al.*, (2000)**
spa	F-TCA ACA AAG AAC AAC AAAATG C R-GCT TTC GGT GCT TGA GAT TC	226	94° C 5 min	94° C 30 sec.	55° C 30 sec.	72° C 30 sec.	72°C 10 min	**Warda *et al.*, (2010)**
(coa)	F-ATA GAG ATG CTG GTA CAGG R-GCT TCC GAT TOT TCG ATGC	Four dif-ferent types of bands may be detected 350 bp 430 bp 570 bp 630 bp	94°C 10 Min.	94° C 1 min.	55° C 1 min.	72° C 1 min.	72°C 10 min.	**Lyer and Kumosani (2011)**
nuc	F-ATATGTATGG-CAATCGTTTCAAT R-GTAAATGCACTT-GCTTCAGGAC	395 bp	94˚C 10 min.	94˚C 45 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Gao *et al.*, (2011)**

Results and Discussion

Results illustrated in tables (2) revealed that 71 out of 120 were proved by culture and bio-chemical tests to be *Staphyloccoci* in addition, 68 out of 71 *Staphyloccoci* isolates proved by coagulase test to be positive with an incidence of (95.8%) mean while the rest three isolates with an incidence of (4.2%) were negative for coagulase test and proved pathogenic by PCR as show in photo (4).

Table (2). Prevalence of *staphylococcus aureus in* the examined buccal swabs of clinical and apparently health dog.

Cases of dogs	No. of isolates	No. of +ve coagulase	Percentage (%)
Clinical cases	48	48	67.6
Apparently health	23	20	28.2
Total	71	68	95.8

The number of owned dogs was increased in the Egyptian society Dogs and become an integral part of households, sharing human lifestyles, bedrooms, and beds, the close contact between household pets and people offers favorable conditions for transmission of bacteria by direct contact (e.g. petting, licking or physical injuries) or indirectly through contamination of food and domestic environ- **(Abdel-Moein & Samir, 2011 and Damborg *et al*., 2016).**

Some dogs will lick their human companions as a show of affection, and thus it would be enlightening to see if companion dogs actually carry MRSA in their mouths as that could potentially be a direct source of transmission. Even if the pet owner was the source of colonization for the dog, the animal would continue to be a possible source of infection if the owner was treated and resolved the infection. Additional studies of this nature could further define the risk factors associated with the transfer of these resistant bacteria from household pets to humans **(Lloyd, 2007)**. It seems more likely that a companion dog will lick the skin or face of an owner as a show of affection rather than rub its nose on the owner. **(Thomas *et al*., 2017**). MRSA Infection in small animals, principally in dogs, has been recorded in many countries, with wound infections, surgical site infections, pyoderma, otitis and urinary tract infections most commonly reported **(Weese and Van Duijkeren, 2010)**, As Table (2) illustrates, that prevalence of *S. aureus* was recovered from (71) dogs swabs samples (59.2%) out of (120) tested swabs. In this study (8) isolates of S. aureus were confirmed to be MRSA for a (11.3%) positive MRSA rate. This result in agreement nearly with **Loeffler *et al*., (2005)** was recorded for *S. aureus* (MRSA) (8.9%) while **Hamadeh *et al.,* (2015)** while **Thomas *et al* (2017)** reported positive rate of MRSA was (3.2%). Staphylococcus aureus (MRSA) isolates

Methicillin Resistant *Staphylococcus aureus* (MRSA) has been recently growing problem in small animal clinical practice due to increasing resistance to multiple number of antimicrobials. **(Sudhakara *et al.,* 2016)**

Table (3). Antimicrobial test results of *Staph. aureus* strains, isolated from buccal samples (71 samples)

Antibiotic	Sensitive		Resistant	
	No.	%	No.	%
Amoxicillin	12	16.9	59	83.1
Ampicillin	14	19.7	57	80.3
Ciprofloxacin	62	87.3	9	12.7
Clindamycin	11	15.5	60	84.5
Doxycyclin	55	77.5	16	22.5
Erythromycin	23	32.4	48	67.6
Levofloxacin	58	81.7	13	18.3
Linezolid	60	84.5	11	15.5
Methicellin	6	8.5	65	91.5
Moxifloxacin	57	80.3	14	19.7
Oxacillin	3	4.2	68	95.8
Penicillin	0	0	71	100
Rifampicin	53	74.6	18	25.4
Tigecycline	56	78.9	15	21.1
Trimethoprim/ Sulfamethoxazole	62	87.3	9	12.7
Vancomycin	7	9.9	64	90.1

in this study were resistance to Amoxicillin, Ampicillin, Clindamycin, Erythromycin, Methicillin, Oxacillin, Penicillin and Vancomycin showed susceptible to Ciprofloxacin, Doxycyclin, Levofloxacin, Linezolid, Moxifloxacin, Rifampicin, Tigecycline and Trimethoprim/Sulfamethoxazoleas, this shows in table (3) these results almost agreed with **Cristina (2012), Abdel-Moein and Samir, (2011) and Ranjekar *et al.* (2015).**

Table (4). Occurance of some virulent and resistant genes mecA, blaz, spa and coagulase in S. aureus isolates from bacal cavity of dogs.

MRSA strain	Aantibiotic resistant genes			Virulent genes		
	mecA	blaZ	tst	spa	Coa	Nuc
1	+	+	-	+	+	-
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	-	+	-	+	-	+
5	+	+	-	+	+	-
6	+	+	-	-	-	-
7	-	+	-	-	-	-
8	+	+	-	+	+	-

The pathogenicity of coagulase positive *staphylococci* are related to the production of many virulence factors including toxins, and enzymes from which coagulase enzyme was consider as the 5 most important one. Coagulase production was described as one of the most reliable criteria for the identification of pathogenic *Staphylococcus* species. *Staphylococci* producing coagulase are usually pathogenic **(Quinn *et al.,* 2002).**

Polymerase chain reaction (PCR)-based methods the simplex and multiplex PCR assays can be used as rapid and sensitive diagnostic tool to detect the presence of *S. aureus*, provide a promising option for the rapid identification made in hours, rather than days consumed by conventional cultural methods **(Mohamed *et al.,* 2015).**

Virulence factors play a key role in disease production by bacterial pathogens among others; their functions include competence, adherence, synthesis, and export of capsules; and evasion of host immune responses **(Nanduri *et al.,* 2009).** The pathogenicity of *S. aureus* has been exacerbated by increased resistance to antibiotics, and methicillin-resistant *S. aureus* (MRSA), encoded by the mecA gene, is associated with increased morbidity and mortality compared to methicillin-sensitive *S. aureus* (MSSA) **(Katayama 2000).** Antimicrobial resistance among *Staphylococci* is based on a wide variety of resistance genes. The most important is methicillin resistance mediated mainly by the mecA gene, which encodes for a penicillin-binding protein (PBP), PBP-2a **(Shore and Coleman 2013)**. In this study mecA gene was detected at 310 bp in 6 (8.5%) isolates out of (8) isolated as in photo (1) and table (4) while **Ashraf, *et al.*, (2018)** recoded 2 isolates harboured mecA at 310 bp. Methicillin resistance is due to the acquisition of the mecA gene, that encodes a new protein designated PBP2a, belonging to a family of enzymes necessary in building the bacterial cell wall. PBP2a has a very low affinity for β-lactam antibiotics and confers resistance to methicillin and the other beta-lactams **(Loeffler *et al.*, 2005 and Pantosti *et al.,* 2007)** Resistance to penicillin is primarily mediated by the blaZ gene, which is responsible for the production of beta-lactamase (penicillinase), an enzyme that hydrolyzes the β-lactam ring of the penicillin molecule. The blaZ gene is part of a transposable element located on a large plasmid, which often carries additional antimicrobial resistance genes, which confer resistance to erythromycin, fusidic acid and gentamicin **(Lowy, 2003).** Our results showed that all 8 (11.5%) tested isolates were positive for blaZ genes at 173bp.as shown in photo (1) and table (4) which means that all isolates harboud mecA gene were positive for blaZ gene. While tst amplification at 326 bp in 2 (2.8%) isolates as photo (3) and table (4).

Typing of the spa gene is also widely used for the epidemiological study of *S. aureus* isolates **(Mathema *et al.,* 2009)**. The spa gene encodes protein A, an important virulence factor of *S. aureus* **(Wright, J.S.).** Among the various DNA sequence-based methods that have been developed to overcome these limitations, spa typing has been shown to be an effective and rapid method for typing MRSA **(Shopsin *et al*., 1999).** In this study amplification of spa gene was detected at 226 bp in 6 (8.5%) isolates out of (8) as in photo (2) and table (4). Also detected amplification of nuc gene at 395bp in 3 (4.2%) isolates out of (8) as in photo (3) and table (4). Several virulence factors produced by *S. auerus* including Coagulase protein which encoded by Coa gene which **is** important in the in the pathogenicity **(Hassan *et al*., 2010).** Through turn fibrinogen to fibrin which lead to abscessiation and persistence of microorganism in host tissue **(McAdow*et al.,* 2011)**. In our study we detected coa gene in 5 (7.0%) isolates and give a single Amplication of 630bp. As shown in photo (4) and table (4).

Identification of *S.aureus resistant gene* using PCR

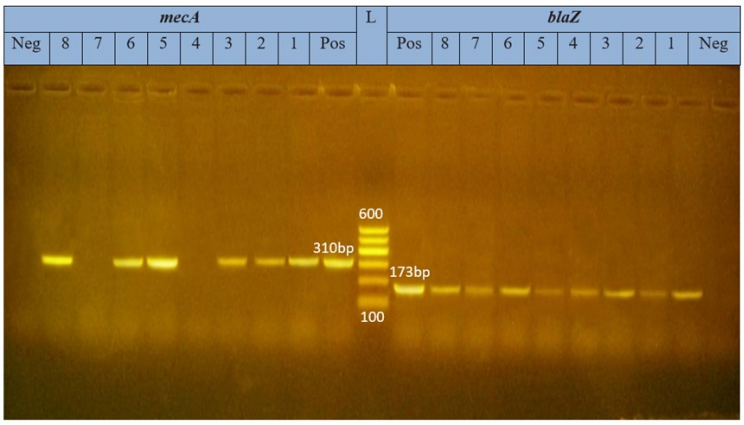

Photo. (1): Illustrated PCR amplification of *S.aureus* isolates extracted DNA for mecA and blaZ genes, L:represents the molecular size marker (100 – 600 bp DNA ladder), N: negative control, P: positive control of mecA (310 bp) and 173 bp for blaZ, samples 1,2,3,5,6 and 8 are positive for mecA gene and samples for 1 to 8 are positive for blaz geng

Detection of *spa* gene of *Staphylococcus aureus*

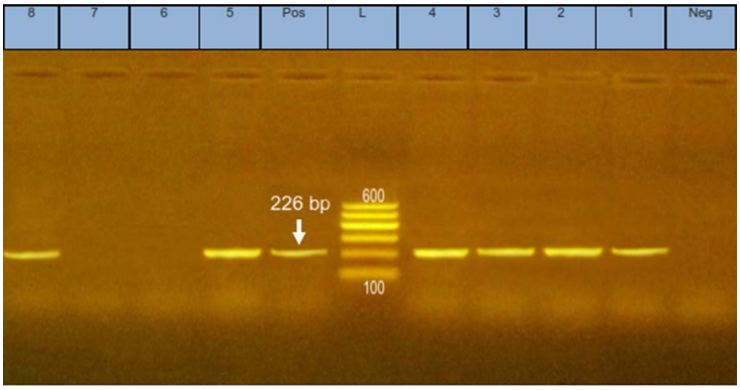

Photo. (2): Illustrated the positive amplification of 226 bp fragment of *spa* gene of *S.aureus* isolates (samples 1, 2, 3, 4, 5 and 8 are positive), N: negative control, P: positive control and L: DNA ladder (100- 600 bp).

Detection of *nuc* gene and *tst* gene of *Staphylococcus aureus*

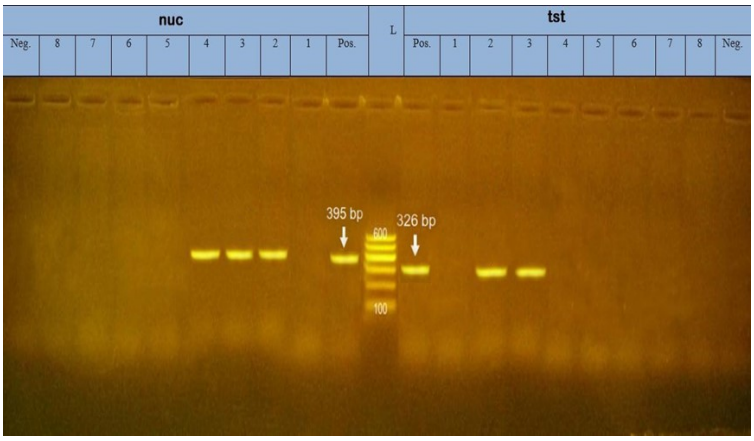

Photo. (3): agarose gel electrophoresis of PCR products after amplification of *nuc* gene at 395.Positive isolates at 395 pb. Lane M: 100-600 pb DNA ladder. Neg: Negative control sample confirmed in CLQP lab to be negative for *nuc* gene). Pos: positive control 395bp. Lane: 2,3,4, pos. Lane: 1,5,6,7,8, Neg. amplification of *tst* gene at 326bp amplified product. Positive isolates at 326 bp.Lane M: 100-600 pb DNA ladder. Neg: Negative control (sample confirmed in CLQP lab to be negative for *tst* gene) Pos: positive control 326pb.Lane: 2,3 pos. Lane: 1,4,5,6,7,8 Neg

Detection of *coa* gene of *Staphylococcus aureus*

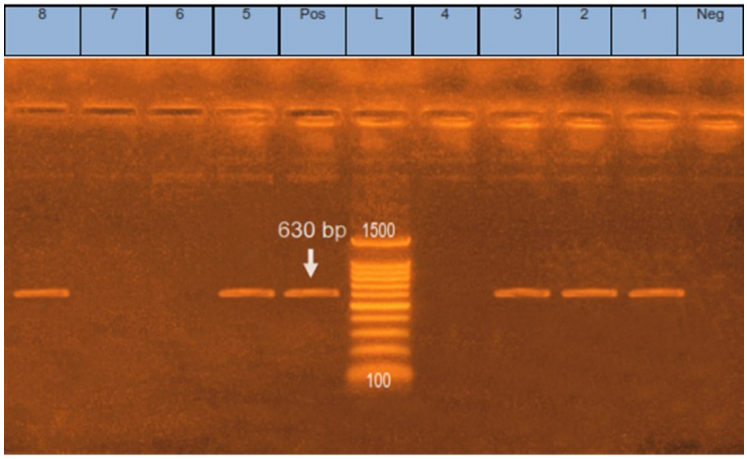

Photo. (4): garose gel electrophoresis of PCR products after amplification of *coa* gene at 630.Positive isolates at 630 pb. *Coa* gene. Lane M: 100-1500 pb DNA ladder. Neg: Negative control (sample confirmed in CLQP lab to be negative for *tst* gene).Pos: positive control 630bp.Lane: 1,2,3, 5 and 8 Pos. Lane: 4,6,7, Neg.

Conclusion and Recommendation

- *Staphylococcus aureus* (MRSA) strains could be transferred to humans through dog bite, Close contact and licking (public health hazard) can also facilitate dog-to-human transmission of the pathogens from their oral cavity. Factors such as immune-suppression, malnutrition and stress that can aid human susceptibility to opportunistic pathogen.
- *Staphylococcus aureus* isolated from oral cavity of dogs are showing multi-drug resistance. These organisms have many virulence and resistance genes which increase their pathogenecity and reduce ability for treatment.
- MRSA colonization is uncommon in healthy pets, if they have been exposed to a hospital environment (such as animals in hospital visitation programs) or to a person who was recently hospitalized, they may be more likely to be carrying MRSA.
- Hand hygiene is the simplest and most practical way to prevent transmission of MRSA and other *S. aureus* strains between humans and animals. Use soap and water or an alcohol-based hand sanitizer on your hands.
- Animals that are positive for MRSA can stay at home if there are no high-risk people (e.g. HIV/AIDS, cancer or transplant patients) in the household. Avoid touching the pet's nose or anal area because these are the most likely areas to harbor MRSA. Don't allow the pet to lick a person's face or any area of broken or damaged skin.

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