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Molecular Divergence of *Staphylococcus aureus* Isolated from Dogs and Cats

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Abstract

Coagulase positive staphylococci particularly *Staphylococcus aureus* are responsible for a variety of illnesses in humans and animals and commonly identified and discriminated by certain virulence factor coagulase (*coa*) genes. A total of 85 nasal discharge and wound samples were collected from 55 dogs and 30 cats. The samples were cultured and examined for detection of *Staphylococcus aureus* and then checked using PCR for the presence of *nuc* and *coa* virulence genes. The positive *coa* bands were subjected to restriction fragment length polymorphism analysis (RFLP) using *AluI* enzyme and sequenced. The results confirmed 10 isolates as *Staphylococcus aureus.* All ten isolates harbored *nuc* and *coa* genes. The RFLP analysis demonstrated five different patterns, and the genetic lineage of recovered isolates was related to a human origin strain sharing 75% homology. This alerts for the potential public health hazard of companion pets.

Keywords: *Staphylococcus aureus*- pets- *coa* gene– *nuc* gene– RFLP

1. Introduction

Staphylococcus aureus could be addressed as an opportunistic pathogen for both humans and animals which is part of the normal microbiota and acts as a pathogen in particular circumstances. Although the bacteria are detected in clinically healthy bodies, they can induce a wide range of infections when the immune system becomes compromised or select conditions are associated (Bierowiec *et al*., 2016). The microbiota on an organism depends on the species, feed, and environment and population density. However, *S. aureus* is the most frequently recovered coagulase positive *Staphylococcus* from the anterior nares and temporarily from the skin of humans (Otto, 2010). Similarly, for dogs and cats; *S. aureus* is also noticed as the major species in this tissue and mucosa's natural microbiota. Investigation of the nasal carriage is used in epidemiology, as an indicator of *S. aureus* exposure with rising risk of infection in humans; among others skin diseases, wound colonization or respiratory tract infections. Also, in pet animals, colonization of the nares with *S. aureus* is commonly used to estimate human exposition to livestock or pet related *S. aureus.* The circulation of *S. aureus* clones varies among hosts, environments and countries (Bierowiec *et al*., 2016).

For a long time, *S. aureus* has been associated with skin, soft tissue infections and foreign body infections. One of the essential virulence figures of *S. aureus* is the unique ability to induce clotting. *S. aureus* secretes two coagulases, an extracellular staphylocoagulase and a cell wall associated von Willebrand factor binding protein that both activate prothrombin to generate fibrin (Peetermans *et al*., 2015). It is clear that coagulase subtyping is useful in *S. aureus* strains discrimination, phylogeny and source tracking through many molecular based techniques. PCR-restriction fragment length polymorphism (RFLP) typing of the coagulase gene (*coa*) can be used to discriminate *S. aureus* strains on the basis of sequence variation within the 3' end coding region of the gene (Ishino *et al*., 2007).

Another virulence factor present in *S. aureus* is the ability of the pathogen to produce nuclease enzyme, which plays an important role in immune evasion by *S. aureus*. It degrades neutrophil extracellular traps, facilitating bacteria to avoid killing and eliminate macrophages from abscesses. Moreover, this enzyme is essential for biofilm, where it is thought to facilitate detachment and dispersal of the biofilm, allowing the bacteria to spread to additional sites (Richard *et al*., 2017).

The companion animals (including cats, dogs) are geographically wide spread and generally not associated with occupational activities. To know the *Staphylococcus* divergence in companion animals is a continuing issue to understand the human – "pets' infection cycle" (Haenni *et al*., 2017).

As these pet species are in frequent and close contact with humans, the epidemiology of *S. aureus* is of public health importance. A better grasp of the molecular mechanisms of *S. aureus* virulence should be a target of promising objective for novel diagnostic and therapeutic strategies. Consequently, this study aimed to the virulence and divergence characterization of *S. aureus* isolated from clinically ill dogs and cats.

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2. Materials and methods

2.1. Sampling and isolation of Staphylococci coagulase positive

A total of 85; 25 nasal and 60 wound swabs were collected from clinically ill dogs (55) and cats (30) in Cairo pet clinics during October 2017- March 2018. The samples were transferred to the microbiology laboratory in National Research Centre, Giza in chilled, insulated containers under aseptic condition. The swabs were incubated in nutrient broth at 37°C for 24 h. Then the samples were subcultured on manitol salt agar (Difco) and incubated at 37°C for 24-48 h, and presumptive coagulase-positive *staphylococci* produce colonies surrounded by bright yellow zones. The presumptive colonies were identified by Gram stain, catalase, coagulase (both the slide and tube) and DNase tests. The isolates were subjected to further testing using API STAPH IDENT 32 Staph (Biomerieux, Marcy l'Etoile, France) (Soriano *et al*., 2000).

2.2. Antimicrobial Susceptibility Testing

Staphylococcus aureus were tested for antimicrobial susceptibility by disc diffusion method in Muller-Hinton agar (Difco) according to CLSI (2015). The following antibiotic discs (Sigma) were used; amoxicillin/clavulinic acid (20/10µg), bacitracin (10µg), gentamycin (10µg), vancomycin (3µg), neomycin (30µg) and polymyxin (10µg).

Susceptibility categorization was carried out according to performance standards for antimicrobial disk susceptibility tests (CLSI 2018).

2.3. Genomic DNA extraction and PCR assay

2.3.1. DNA extraction

The standard *S. aureus* strain (ATCC 25923) was obtained from the reference laboratory of the Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain-Shams University.

DNA was extracted from *S. aureus* isolates and the standard strain using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 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 100 µl of elution buffer provided in the kit.

2.3.2. PCR detection of nuc gene and coa gene

For either *nuc* or *coa* uniplex PCRs were carried out in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The *coa* gene was amplified using two sequences of primers: 5′-CGAGACCAAGATTCAACAAG-3′ and 5′-AAAGAAAACCACTCACATCA-3′. For the amplification of *nuc* gene were used the following primers: R: 5′ GTTTTTGGCTGCTTCTCTTG-3′ and F: 5′-ATATGTATGGCAATCGTTTCAAT-3′. The primers used were supplied from Metabion (Germany). Amplification was conducted in thermal cycler (Biometra, Germany), which was adjusted for *coa* gene amplification as follows (Aslantaş *et al*., 2007): an initial denaturation at 94˚C for 3 min, 30 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, and extension at 72˚C for 1 min and a final extension at 72˚C for 5 min. For amplification of *nuc* gene the program consisted of an initial denaturation at 94˚C for 4 min, 30 cycles of denaturation at 94˚C for 30 sec, annealing at 50˚C for 30 sec, and extension at 72˚C for 1 min and a final extension at 72˚C for 4 min (Gao *et al*., 2011). The amplification of *coa* and *nuc* genes generated amplicons of 970 bp and 270 bp respectively.

The PCR reaction mixtures were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of 100- bp DNA ladder (Fermentas Life Science, EU) according to (Sambrook *et al*., 1989).

2.4. Coagulase gene typing by RFLP method

S. aureus strains positive for *coa* gene were subjected to restriction fragment length polymorphism. PCR amplified *coa* gene products were digested with *AluI* enzyme (Aslantaş *et al*., 2007). Restriction master mix was prepared according to Promega instruction kit, accurate fragment size analysis based on the electrophoretic mobility and was determined by using DNA size TOTALAB 1D analysis software. For sequencing of PCRs' products each amplicon was purified using the ExoSAP-IT PCR Product Cleanup kit (Affymetrix, USA) according to the manufacturer's instructions. Sequencing reactions were performed with the ABI PRISM®BigDyedye™ terminator cycle sequencing kit with AmpliTaq® DNA polymerase on an MJ Research PTC-225 Peltier Thermal Cycler (Applied Biosystems, USA) as described by the manufacturer. Each sequencing reaction was repeated at least 3 times in both directions before being accepted for analysis. Then sequences of each PCR product were aligned with homologous GenBank records (http://www.ncb i.nlm.nih.gov) by multiple sequence alignment using the Clustal®W program46.

3. Results

The conventional cultural, staining methods showed an isolation of a total number of 18 *staphylococci* (table 1). Fourteen (23.3%) were isolated from wound swabs (8 from dog samples and 6 from cats) and the rest 4 (16%) were isolated from nasal swabs (3 from dogs while the last one from cat source).

Table 1. Number of *Staphylococci* isolated from dog and cat samples.

Isolation origin	No. of *Staphylococci* isolates / wound swabs	%	No. of *Staphylococci* isolates / nasal swabs	%	Total No. of isolates / total No. of swabs	%
Dog	8 / 35	22.8	3 / 20	15	11/ 55	20
Cat	6 / 25	20.8	1/ 5	16.6	7 / 30	22.2
Total	14 / 60	23.3	4 / 25	16	18 / 85	21.2

Considering the coagulase production species specific API identification, 10 isolates were confirmed as *S. aureus* (table 2). Seven were from dog samples (5 from wounds and 2 from nasal swabs) while the other three of feline source (2 from wounds and the last one from nasal swabs).

Table 2. Number of confirmed *S. aureus* isolates.

No. of confirmed *S. aureus* isolates	wound swabs	nasal swabs	Total
Dog	5	2	7
Cat	2	1	3
Total	7	3	10

The susceptibility of the isolates to six antibiotics was achieved using the disc diffusion method. According to this technique *S. aureus* isolates were resistant to bacitracin, gentamycin and neomycin 100% and more sensitive to amoxicillin/clavulinic acid and vancomycin 94, and 88% respectively.

All *S. aureus* isolates showed positive *nuc* gene amplification using specific primer and produced amplicons of average molecular size 270 bp (figure 1). In the same way, all *S. aureus* isolates showed positive *coa* gene amplification and produced the amplicons of 970 bp (figure 2).

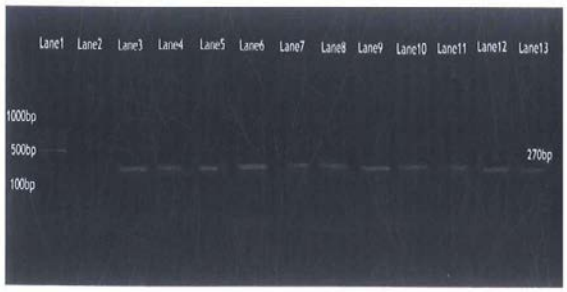

Figure 1: Amplified PCR product of *nuc* gene Lane 1: 100bp ladder. Lane 2: negative control. Lane 3: positive control. Lanes 4-13: represented *S. aureus* isolates, the *nuc* gene PCR products of 10 confirmed *S. aureus* produced same sized fragments 270 bp.

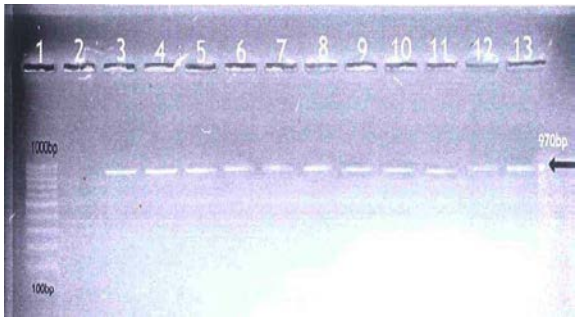

Figure 2: Amplified PCR product of *coa* gene Lane 1: 100bp ladder. Lane 2: negative control. Lane 3: positive control. Lanes 4-13: represented *S. aureus* isolates, the *coa* gene PCR products of 10 confirmed *S. aureus* produced same sized fragments 970 bp.

The *coa* gene PCR products (970 bp) were exposed to restriction digestion using RFLP with *AluI* enzyme. The fragment size was accurately analyzed by using DNA size TOTALAB 1D analysis software. According to figures 3 and 4, and table 3, a total of 5 different electrophoretic patterns of the confirmed 10 isolates were obtained (I, II, III, IV and V), with predomination of pattern III in 3 dog wound samples, and the patterns tend to be specific.

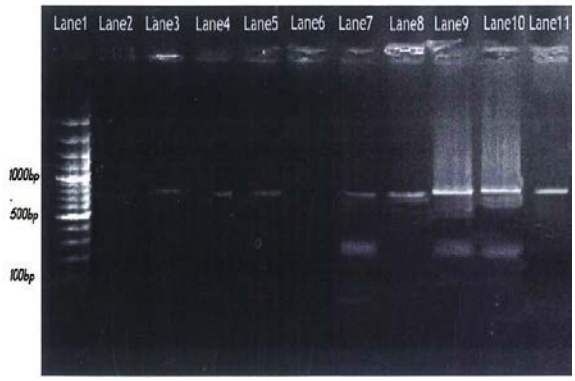

Figure 3. Restricted fragments of *coa* gene by *AluI* enzyme; Lane 1: 100bp ladder. Lanes2,3: represented dog nasal samples isolates. Lanes (4-8) represented dog wound samples isolates, Lanes (9,10) represented cat wound samples isolates. Lane 11represented cat nasal sample isolate. The results of *coa* gene typing of *S. aureus* isolates by RFLP were mentioned in the table (4).

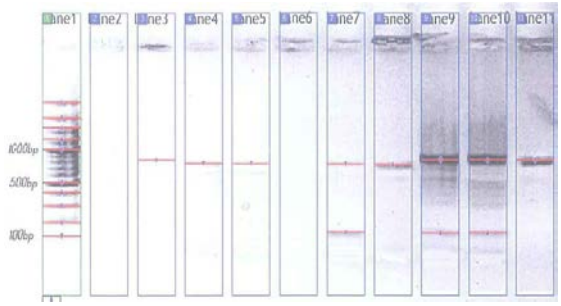

Figure 4: ID software analysis of *coa* gene restriction products.

Table 3. Restricted fragment size of *coa* gene, genotypes and sample sources.

Sample no	Source	Restricted fragments (bp)	Genotype
1(lane 2)	Dog nasal	-	I
2 (lane 3)	Dog nasal	825	II
3 (lane 4)	Dog wound	795	III
4 (lane 5)	Dog wound	795	III
5 (lane 6)	Dog wound	-	I
6 (lane 7)	Dog wound	105, 795	IV
7 (lane 8)	Dog wound	795	III
8 (lane 9)	Cat wound	105, 825	V
9 (lane 10)	Cat wound	105, 825	V
10 (lane 11)	Cat nasal	825	II

Sequences of the amplified *coa* gene exhibited a (99-100%) consistency when matched with those obtainable in GenBank using gapped BLASTN software. Identification to the species and subspecies levels was considered for segments with sequences identities >97%. BLASTN analysis of the ranged sequences of the isolates showed 96-99% identity with the similar genes sequence of *S. aureus* subsp. *aureus* strain M013 (accession No. CP003166).

4. Discussion

Staphylococcus genus includes up to 58 valid species, and subspecies, as two species *S. argenteus* and *S. schweitzeri* were identified (Tong *et al*., 2015), and another new coagulase negative species; *S. edaphicus* has been recovered from Antarctica (Pantůček *et al*., 2017).

Coagulase positive *Staphylococcus* (CPS) such as *S. aureus* includes both human and animal pathogens and is considered the major player associated with outbreaks of food intoxication worldwide (Argudin *et al.*, 2010; Hennekinne *et al*., 2012). In Egypt, many studies highlighted the role of CPS as animal pathogens. The frequent isolation of CPS from mastitic milk of cows and buffaloes were encountered (El-Jakee, 2008; El Seedy *et al.*, 2012).

Moreover, the isolation of CPS from septic wound and infected respiratory tract has drawn the attention to the risk of this pathogenic bacteria to human beings and consequently to pet animals, who are in a continuous contact with humans and act as reservoir of coagulase positive *Staphylococcus* (Robinson, 2004). It has been shown previously that *S. aureus* colonization of the nose increases the risk for developing a *S. aureus* infection (Wertheim *et al.*, 2005). Bacteremia is more common in the severe cases of skin infections but even the mild superficial cases carry a risk of systemic spread. Therefore, skin and soft tissue infections are the most commonly reported sources of systemic bacteremia (Wilson *et al*. 2011). All such aspects support that the identification of *S.aureus* is crucial for proper management of skin infection abscesses, septicemia/bacteremia or respiratory infections of pet animals which in contact to humans gain public health importance (Jurate and Jurate 2015).

In the present study, the data obtained (table 2) demonstrated the isolation of 18 *staphylococcus* with an average incidence of 20-23%. The confirmed *S. aureus* isolates constituted little more the half number 10 as the wounds showed 7 isolates (5 from dogs and 2 from cats) while nasal swabs exhibited 3 isolates (2 from dogs and one from cats). The nearly same incidences were reported in other studies, 24.58% (Abraham *et al.*, 2007) and 22.9% (Jang *et al.*, 2014). Higher incidences also were detected 36.3% (Drougka *et al.*, 2016; Han *et al*., 2016). The high rate of *staphylococci* recovery is of particular zoonotic importance, as such pet animals can constitute a dangerous source of infection to pet owners (Bierowiec *et al*. 2014).

The susceptibility of *S. aureus* to antimicrobials was tested by agar diffusion method. The results showed that the isolates were resistant to bacitracin, gentamycin and neomycin and more sensitive to amoxicillin/clavulinic acid and vancomycin. Similar findings were obtained by Hakim *et al*. (2016) and Conner *et al*. (2018), however in another study *S. aureus* was susceptible to bacitracin (89.3%) and gentamicin (86.1%) (Chang *et al*., 2015).

Thermonuclease (*nuc*) gene is used as primary molecular target for rapid identification of *S. aureus* as an important characteristic virulence factor of the pathogen (Thomas *et al.*, 2007). The results obtained (figure 1) showed that all examined isolates produced amplicons of same average size 270 bp. The same results were mentioned in many studies; Balbutskaya *et al*., (2017) confirmed 100% of 117 *S. aureus* isolates for *nuc* molecular analysis. Also, Jiang *et al*., (2019) detected *nuc* gene in all *S. aureus* investigated isolates. Hoegh *et al*. (2014) also mentioned that the occurrence of *nuc*-negative *S. aureus* isolates is extremely rare, representing less than 1% of all *S. aureus* isolates.

Production of coagulase is an important phenotypic feature, used worldwide to identify *S. aureus*. Several studies have implemented the molecular analysis of the coagulase gene as an accurate defined test (Tiwari *et al.* 2008).

In our study, all examined *S. aureus* isolates amplified *coa* gene, producing an amplicon of 970 bp. An uniform amplicon of 500bp was mentioned by another study among *S. aureus* subclinical mastitis strains in Nigeria (Suleiman *et al*., 2012).

This result may be unusual; as commonly, the *coa* gene encoding coagulase protein is highly polymorphic because of the inconstant sequences (81 bp tandem repeats) at its 3' end (Janwithayanuchit *et al*., 2006). There is a great variability in size of a *coa* gene on cited articles in that topic as many authors studied variation of the obtained PCR product for *coa* gene by using the same primer (Salasia *et al.*, 2004; Kalorey *et al.*, 2007; Reinoso *et al.*, 2008; Hakim *et al.*, 2017).The *coa* gene polymorphism allows differentiation of *S. aureus* species. Depending on this phenomenon, it has been considered a technically simple method of good reproducibility and discriminatory power for typing *S. aureus* strains (Javid *et al*., 2018).

The cause for this polymorphism among *S. aureus* isolates is indistinct, but it seems to be due to deletion or insertion of many nucleotides at 3' end region. This results in mutations and a consequent change of the *coa* gene size and perhaps antigenic properties of the coagulase enzyme (Saei *et al.*, 2009). This antigenic variation of coagulase is supposed to be essential for the pathogen escape from inhibitory effect of anticoagulase components. Some reports have suggested that antibody and non-antibody agents can neutralize the coagulase activity and therefore increase resistance against staphylococcal infections (Engelmann and Massberg 2013).

According to figures 3 and 4, a total of 5 different electrophoretic patterns (I, II, III, IV and V) from the 10 confirmed *S. aureus* isolates were observed. The patterns tend to be specific with predominance of pattern III in 3 dog wound samples, followed by pattern I and II. The pattern V was present in 2 cats wound samples while the pattern IV was observed in one dog wound sample.

Although many genotypes were detected, only a few prevailed. This perception was frequently observed; in an earlier survey performed in Brazil, the 64 *S. aureus* isolates were categorized into 49 types for *coa* gene RFLP with the 10 most observed representing 39% of the isolates (da Silva and da Silva, 2005). In other studies, PCR-RFLP of *coa* gene differentiated the isolates into 15 genotypic patterns, of which 4 patterns only were predominant (Sharma *et al.*, 2017).

Sequencing of staphylococcal *coa* genes obtained from the ten *S. aureus* isolates was performed during this study. Gene sequences exhibited a high level of inter-individual correspondence (99-100%) confirming that the amplified products were really identical to the *coa* gene. The *coa* gene is a target for phylogenetic analysis of staphylococci and differentiation of staphylococcal isolates at the species level. Canine and feline isolates came in one clade and

shared homology by 75% with the strain obtained in 2002 from a wound specimen of a pediatric outpatient in Taiwan (Huang *et al*., 2012).

5. Conclusion

The analysis of the *coa* gene supplied a useful typing tool for *S. aureus* from various pet animals. Additionally, the obtained data showed that the studied cases harbored *S. aureus* isolates and that more than one coagulase genotype was detected, although only one or two forms dominated. However, further studies using a large collection of strains from different areas and animals are needed so that the obtained information improves the efficiency of staphylococcal infection control measures. Moreover, these results assume the probability of the transmission of some strains of coagulase positive staphylococci in between human and companion pets.

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