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International Journal of Current Research
Vol. 10, Issue, 04, pp.67597-67605, April, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

MOLECULAR DETECTION OF VIRULANCE *NUC* GENE OF *STAPHYLOCOCCUS* SPECIES ISOLATED FROM SINUSITIS'S PATIENTS BY MULTIPLEX PCR

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ARTICLE INFO

Article History:
Received 27th January, 2018
Received in revised form
05th February, 2018
Accepted 28th March, 2018
Published online 30th April, 2018

Key words:
coagulase positive *Staphylococcus*, coagulase negative *Staphylococcus*, *Staphylococcus* species, sinusitis

ABSTRACT

The staphylococci are most frequently isolated from clinical specimens in the microbiology laboratory. These bacteria are widespread in nature and can be recovered from environment or as commensally inhabitants of the skin, mucous membranes and other body sites in humans and animals. Aim of the current study was to Identify the different coagulase positive (CoPS) and coagulase negative (CoNS) *Staphylococcus* species includes *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* isolated from sinusitis's patients by multiplex PCR. In this study nasal swabs from 150 patients were used for culture, VITEK 2 system and multiplex PCR analysis. Multiplex PCR was performed with species-specific primers targeted to the *nuc* gene of *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis*. The result of the multiplex PCR was compared with conventional culture,VITEK 2 system methods. The positive multiplex PCR product was identified by presence of ~359 bp, ~659 bp, ~251 bp and ~ 177 amplicons of *nuc* gene for the *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis*, reaspectively. Conventional methods of *Staphylococcus* culture, VITEK 2 system, showed that to sum up 100 out of 150 nasal swabs were detected for *Staphylococcus* species; To sum up 31 out of 100 nasal swabs were detected for *S. aureus*, 16 out of 100 nasal swabs were detected for *S. lugdunensis,* 10 out of 100 nasal swabs were detected for *S. epidermidis,* 6 out of 100 nasal swabs were detected for *S. hominis* and 37 out of 100 nasal swabs were detected for other *Staphylococcus* species. Five of the 60 samples that were negative by conventional methods were positive by multiplex PCR. Statistical analysis revealed that the PCR to have a sensitivity of 95.5 % in the detection of *Staphylococcus* species in sinusitis. This multiplex PCR method provides a sensitive, rapid, and reliable alternative to conventional methods to identify *Staphylococcus* species isolated from patients clinically diagnosed with sinusitis.

Citation: Maryam N. Hamad and Saife D. Al-Ahmer, 2018. "Molecular detection of virulance *nuc* gene of *staphylococcus* species isolated from sinusitis's patients by multiplex pcr", *International Journal of Current Research*, 10, (04), 67597-67605.

INTRODUCTION

The staphylococci are most frequently isolated from clinical specimens in the microbiology laboratory with the exception of the *Enterobacteriaceae*. These bacteria are widespread in nature and can be recovered from environment or as commensally inhabitants of the skin, mucous membranes and other body sites in humans and animals (koneman *et al*., 1988). *Staphylococcus aureus* causes pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis, endocarditis and superficial skin lesions such as furunculosis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* also causes food poisoning by releasing enterotoxins in to food, and toxic shock syndrome (Quinn *et al*., 1994). Coagulase negative staphylococci (CNS) are associated with the normal skin flora and mucous membranes and can be isolated from many other sources such as meat, milk and cheese, soil, sand, water, and air (Kloos and Bannerman, 1994). CNS may cause bacteremia, endocarditis, catheter related infections, central nervous system shunt infections, urinary tract infections, endophthalmitis, and infections of prosthetic joints. CNS give rise to significant hospital infections often associated with the use of medical devices and immunocompromised patients (Huebner and Goldmann, 1999). Unlike other CNS*, S. lugdunensis* is highly pathogenic and can cause aggressive skin and soft tissue infections, bone and joint infections, and native valve endocarditis (Frank *et al*., 2008; Rogers *et al*., 2009). In addition, the oxacillin MIC breakpoints to determine methicillin resistance differ among staphylococcal species. Therefore, it is important to identify staphylococci at the species levels (von Eiff *et al*., 2002). Staphylococcal nuclease is considered as an important virulence factor and a unique marker widely used in the detection of *Staphylococcus aureus*

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from food samples and clinical specimens (Alarcon *et al.,* 2006). It is an exoenzyme that could hydrolyze DNA and RNA in host cells, causing tissue destruction and spreading of *S. aureus* (Sandel and McKillip, 2004). It was also the first indication of new class of sugar-nonspecific nucleases because of its high level of extracellular production and remarkable heat stability in *S. aureus* (Foster, 2005). Sugar non-specific nucleases are characterized by their ability to hydrolyze both DNA and RNA without exhibiting pronounced base preferences (Rangarajan and Shankar, 2001). They play important roles in DNA and RNA degradation, which is essential in microbial defense mechanisms and programmed cell death (Hsia *et al.,* 2005; Parrish and Xue, 2006). Sequence analyses of several target genes are the most reliable methods (Kwok and Chow, 2003). However, these are expensive, laborious, and time-consuming. Thus, a simple and reliable assay is needed for identification of staphylococcal species (Poyart *et al*., 2001; Drancourt and Raoult, 2002; Shah *et al*., 2007). Modern molecular biological techniques for the detection and differentiation of pathogens gain more and more importance. Amplification of thermonnusclease (*nuc*) gene is used to detect and quantify and known as species specific gene (Studer *et al.,* 2008). Real-time PCR for detection of pathogenic bacteria as species and others has properties of high specificity and sensitivity, and can be completed in one day (Ching-Yang *et al.,* 2012).

MATERIALS AND METHODS

Samples collection: Samples were obtained from patients clinically diagnosed with sinusitis in four hospitals at Baghdad which are; Baghdad Teaching Hospital-Medical city, Al-Kadhmiya General Teachining Hospital, Al-Yarmuk General Teaching Hospital, and Al-karkh Hospital.for a period of 3 months. One hundred fifty cases of sinusitis were investigated in this study. Patients' ages ranged from 20 to 40 years of age. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq. Nasal swabs were obtained from 150 patients clinically diagnosed with sinusitis by gynecologist with a sterile swabs and cultured on nutrient agar and blood agar (Himedia/ India) at 37°C for overnight under aerobic condition, then the positive cultures of *Staphylococcus* species on were cultured on mannitol salt agar at (Salucea/ Netherland) at 37°C for overnight under aerobic condition also, as deferential medium for coagulase positive and coagulase negative staphylococci such as *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* from other *Staphylococcus* species. Biochemical test and VITEK 2 system were used as a conventional diagnosis for the *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* than other *Staphylococcus* species.

DNA extraction: Genomic DNA was extracted from the *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modifications. Briefly, 1 ml culture of *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* isolates grown for 24 hours at 37°C in nutrient broth (Sigma, USA) were transferred to a 1.5 ml microcentrifuge tube. The samples were centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed, then the cells were resuspended thoroughly in 300 µl of 50 mM EDTA (Sigma, USA). 60 µl of 20 mg/ml proteinase K (Sigma, Missouri, USA) was added and gently pipet 4 times to mix, then the samples were incubated at 37°C for 30 minutes to digest the cell wall and cooled at room temperature. The samples were centrifuged at 13,000 rpm for 3 minutes, the supernatant was removed and 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added to the cell pellet, then gently pipet to mix. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added and vortex vigorously at high speed for 20 seconds, then the samples were sit on ice for 5 minutes and centrifuged at 14,000 rpm for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 ml microcentrifuge tube containing 800 µl of cold absolute ethanol and gently mixed by inversion until the thread-like strands of DNA form a visible mass, then centrifuged at 14,000 rpm for 10 minutes.

The supernatant was carefully decanted and the tubes were drained on clean absorbent paper and 600 µl of room temperature 70% ethanol were added, then the tubes were gently inverted several times to wash the DNA pellet. The samples were centrifuged at 14,000 rpm for 2 minutes and all the ethanol was carefully aspirated. The tubes were drained on clean absorbent paper and the pellet was allowed to air-dry for 10 minutes, then 50 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added. 1.5 µl of RNase solution (wizard genomic DNA purification kit) was added to the purified DNA sample and the sample was vortex for 1 second, then centrifuged briefly in a microcentrifuge for 5 seconds to collect the liquid and incubated at 37°C for 15 minutes. The DNA was rehydrated by incubating at 65°C for 1 hour and the solution was periodically mixed by gently tapping the tube, then the DNA sample was stored at -20°C until use.

DNA quantification: The extracted DNA from the *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* isolates were quantified spectrophotometrically at OD260/280 nm with ratios 1.3-1.6. The sensitivity of the (aur-F, aur-R), (lug-F, lug-R), (epi-F, epi-R) and (hom-F, hom-R) primers were evaluated by PCR amplification for serial diluted concentrations (10ng-100ng) of purified genomic DNA isolated from *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis,* respectively.

Multiplex PCR primers selection: The species-specific primers for *nuc* gene of *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* as the target gene for this study were selected according to (Hirotaki *et al*., 2011). These sets of unique primers were designed based on the conserved regions in *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* primers were synthesized by Alpha DNA, Kanda. The name, sequence and expected product size of these species-specific primers for *nuc* gene are shown in (Table 1).

Multiplex PCR Master Mix: The multiplex PCR reaction for detection of *nuc* gene for *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* was performed in 50 µl volumes containing 6 µl of nuclease free water, 20 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 1 µl of 20 pmol aur-F, aur-R primers, 1 µl of 20 pmol lug-F, lug-R primers, 1 µl of 20 pmol epi-F, epi-R primers, 1 µl of 20 pmol hom-F, hom-R primers, and 4 µl of genomic DNA sample of each *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis*, The mixes were overlaid with 2 drops of mineral oil.

Table 1. Name, sequence and the expected product size of *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* primers.

Staph. species	Name of primer	Sequence of primer	Expected product size (bp)
S. aureus	aur-F	5'-TCGCTTGCTATGATTGTGG-3'	~359
	aur-R	5'- GCCAATGTTCTACCATAGC-3'	
S. lugdunensis	lug-F	5'-TCCAATGATGGTAACGAGGC-3'	~659
	lug-R	5'- TTTTGCGCCTCGTTTTGTCG-3'	
S. epidermidis	epi-F	5'-TTGTAAACCATTCTGGA CCG-3'	~251
	epi-R	5'- ATGCGTGAGATACTTCTTCG-3	
S. hominis	hom-F	5'-TACAGGGCCATTTAAAGACG-3'	~177
	hom-R	5'- GTTTCTGGTGTATCAAC ACC-3'	

Multiplex PCR program: Multiplex PCR was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Hirotaki *et al*., 2011). Briefly, the Amplification of *nuc* gene for was carried out *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* with initial denaturation at 96°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for (aur-F, aur-R), (lug-F, lug-R), (epi-F, epi-R) and (hom-F, hom-R) primers for 30 seconds, and extension at 72°C for 1 minute. The thermal cycles were terminated by a final extension for 5 minutes at 72°C.

Multiplex PCR products analysis: The analysis of multiplex PCR product of *nuc* gene for *S. aureus, S. lugdunensis, S.epidermidis,* and *S. hominis* was performed on 1% agarose gels. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The PCR product was stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

RESULTS

Culture on nutrient agar: All the nasal swabs that collected from the patients clinically diagnosed with sinusitis were cultured on the nutrient agar plates and the morphological characteristics of these growing isolates were determined. The results showed grow of creamy colonies of *S. aureus* isolates (Figure 1), and white colonies of *S. lugdunensis* isolates (Figure 2), whereas the *S. epidermidis i*solates showed grow of creamy colonies (Figure 3) and the *S. hominis* isolates gave white colonies (Figure 4) on nutrient agar plate.

Figure 1. Creamy colonies of *Staphylococcus aureus* grow on nutrient agar plate

Culture on blood agar: Also, all the nasal swabs that collected from the patients clinically diagnosed with sinusitis were cultured on the blood agar plates and the morphological characteristics of these growing isolates were determined. The results showed grow of round and white-gray colonies of *S. aureus* isolates (Figure 5), *S. lugdunensis* isolates (Figure 6), *S. epidermidis* isolates (Figure 7) and *S. hominis* (Figure 8) isolates on blood agar plate.

Figure 2. White colonies of *Staphylococcus lugdunensis* isolate grow on nutrient agar

Figure 3. Creamy colonies of *Staphylococcus epidermidis* isolate grow on nutrient agar

Figure 4. White colonies of *Staphylococcus hominis* isolate grow on nutrient agar

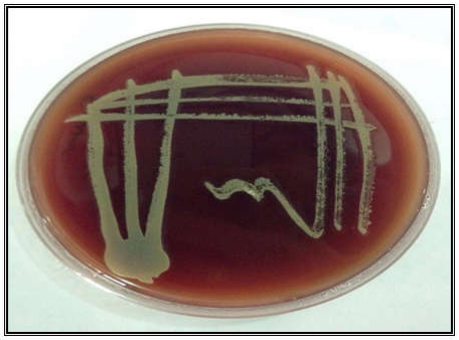

Figure 5. Gray colonies of *Staphylococcus aureus* isolate grow on blood agar plate

Figure 6. Gray colonies of *Staphylococcus lugdunensis* isolate grow on blood agar plate

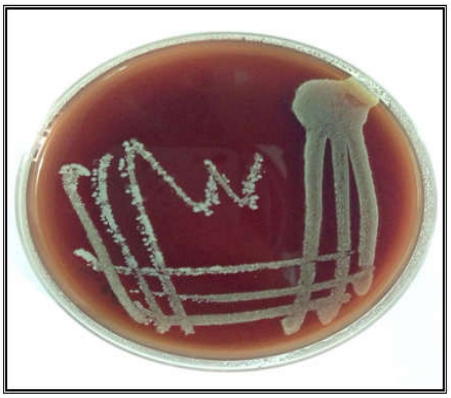

Figure 7. Gray colonies of *Staphylococcus epidermidis* isolate grow on blood agar plate

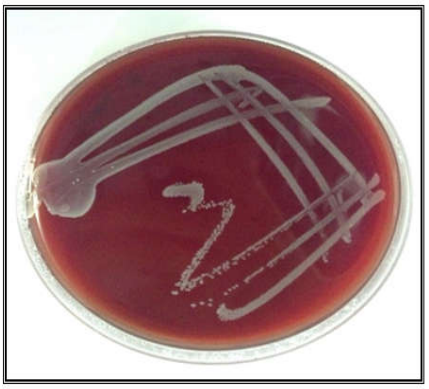

Figure 8. Gray colonies of *Staphylococcus hominis* isolate grow on blood agar plate

Culture on mannitol salt agar: In addition, all the nasal swabs that collected from the patients clinically diagnosed with sinusitis were cultured on the mannitol salt agar plates and the morphological characteristics of these growing isolates were determined.

Figure 9. Golden colonies of *Staphylococcus aureus* isolate grow on mannitol salt agar plate.

Figure 10. Creamy colonies of *Staphylococcus lugdunensis* isolate grow on mannitol salt agar plate.

Figure 4-11. Pink colonies of *Staphylococcus epidermidis* isolate grow on mannitol salt agar plate

Figure 12. White colonies of *Staphylococcus hominis* isolate grow on mannitol salt agar plate

The results showed grow of golden colonies of *S. aureus* isolates (Figure 9), and creamy colonies of *S. lugdunensis* isolates (Figure 10), whereas the *S. epidermidis* isolates showed grow of light pink colonies (Figure 11), and the *S. hominis* isolates gave white colonies (Figure 12).

VITEK 2 system: The VITEK 2 system was used to diagnosis the *S. aureus, S. lugdunensis, S. epidermidis,* and *S. hominis* isolates of this study. At the end of the incubation period, VITEK 2 system was identified presence of *S. aureus* in 31 samples (31%), *S. lugdunensis* in 16 samples (16%), *S. epidermidis* in 10 samples (10%) and *S. hominis* in 6 sample out of 100 positive culture samples of nasal swabs that were collected from patients clinically diagnosed with sinusitis . Whereas the other 37 samples (37%) out of 100 positive culture samples of nasal swabs were identified as other *Staphylococcus* species.

Analysis of extracted DNA: After performing of DNA extraction from *S. aureus, S. lugdunensis, S. epidermidis* and *S. hominis* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of extracted DNA of *S. aureus, S. lugdunensis, S. epidermidis* and *S. hominis* using 1% agarose gel at 7volt /cm for 1 hour (Figure 13), (Figure 14), (Figure 15) and (Figure 16) respectively.

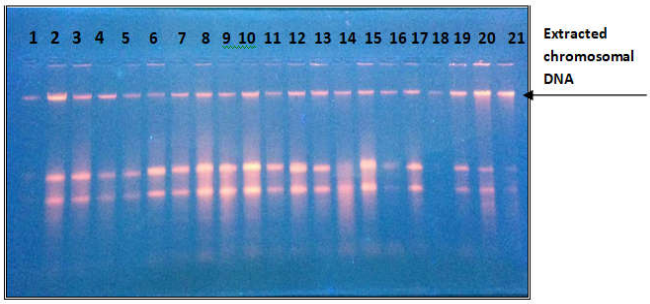

Figure 13. Gel electrophoresis of extracted DNA of *S. aureus* using 1% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-6: Extracted DNA.

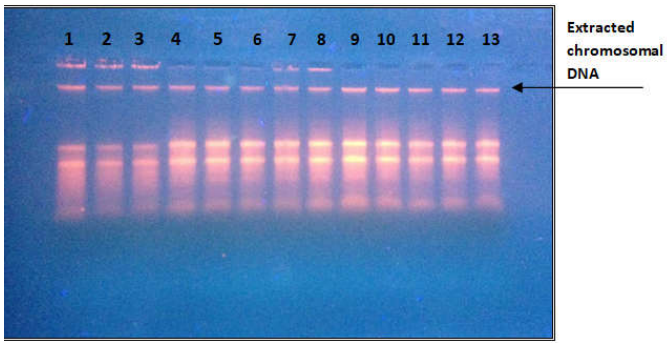

Figure 14. Gel electrophoresis of extracted DNA of *S. lugdunensis* using 1% agarose gel at 7volt /cm for 1 hour. Lane 1-10: Extracted DNA.

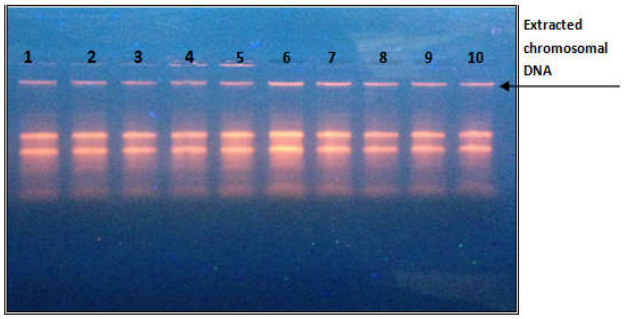

Figure 15. Gel electrophoresis of extracted DNA of *S. epidermidis* using 1% agarose gel at 7volt /cm for 1 hour. Lane 1-6: Extracted DNA

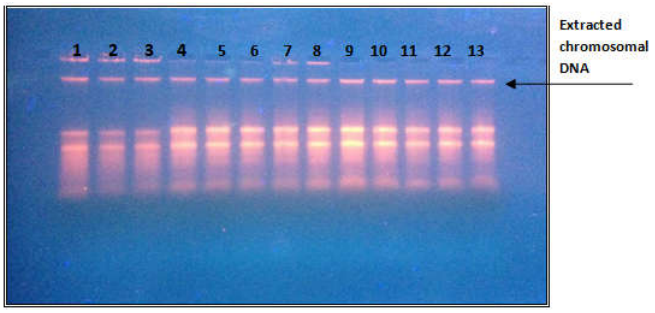

Figure 16. Gel electrophoresis of extracted DNA of *S. hominis* using 1% agarose gel at 7volt /cm for 1 hour. Lane 1-6: Extracted DNA

Analysis of multiplex PCR products: In successful multiplex PCR reaction, the *nuc* gene products of ~ 359 bp, ~695 bp, ~251 bp and ~177 bp molecular weights for *S. aureus, S. lugdunensis, S. epidermidis* and *S. hominis* were observed respectively. This was considered as mandatory sign of successful reaction; upon gel electrophoresis, the bands were located between 300 to 400 bp, 600 to 700 bp, 200 to 300 bp and 100 to 200 bp bands of the 100 bp DNA ladder respectively, indicating the presence of this gene in these *Staphylococcus* species. In the 100 patients clinically diagnosed with sinusitis, to sum up 31 (31%) out of 100 samples were detected for *S. aureus,* 16 (16%) samples for *S. lugdunensis,* 10 (10%) samples for *S. epidermidis* and 6 (6%) samples for *S. hominis*, and 37 (37%) out of 100 samples were detected for other Staphylococcus species. The PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5 µl of the PCR product were loaded on 1.5% agarose gel and run at 7volt /cm for 1 hour. The gel was stained with ethidium bromide solution (0.5µg/ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system (Figure 17).

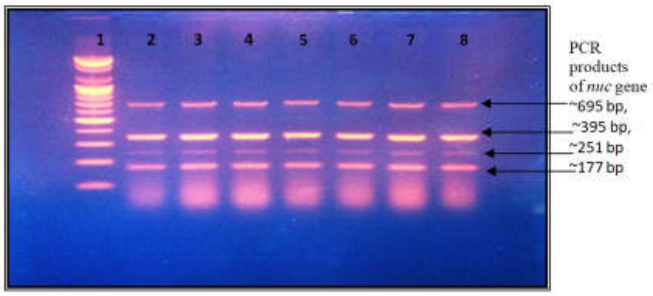

Figure 17. Gel electrophoresis of multiplex PCR product of *nuc* gene for *S. aureus, S. lugdunensis, S. epidermidis* and *S. hominis* isolates using 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-10: multiplex PCR products of *nuc* gene

DISCUSSION

Isolation of *S. aureus, S. lugdunensis, S. epidermidis* and *S. hominis*: In this study, the result of *Staphylococci* species isolation showed that the first most common isolated species was *S. aureus,* the second isolated species was *S. lugdunensis*, the third isolated species was *S. epidermidis,* the fourth isolated species was *S. hominis* and the other samples were for other *Staphylococci* species that isolated from patients clinically diagnosed with sinusitis in this study. The result of this study in agreement with results of different worldwide studies which conducted on *Staphylococci* species that referred the first and second most common isolated species were *S.*

aureus and *S. lugdunensis* (Wertheim *et al.,* 2004; Lebon *et al.,* 2010; Skramm *et al.,* 2011; Sangvik *et al.,* 2011). Also, the result of *S. aureus* isolation goes together with results of different worldwide studies associated with *S. aureus* isolation showed that the percentage of isolation for *S. aureus* was (36.4%) in Switzerland (Mertz *et al.,* 2009), from the National Health and Nutrition Examination Survey (NHANES) in USA the prevalence was reported to be (30.4%) for *S. aureus* in the period 2001-2004 (Gorwitz *et al.,* 2008). Similar rates were found among Japanese volunteers showing a nasal carriage of (35.7%) for *S. aureus* (Uemura *et al.,* 2004), In Mexico was reported to be (37.1%) (Hamdan-Partida *et al.,* 2010), while within a remote adult population in the Amazonian forest the overall carriage was as high as (41.7%) in 2006 and (57.8%) in 2008 (Ruimy *et al.,* 2010). Whereas, the result of this study partially disagree with results of other studies conducted in South and Southeast Asia that exhibited quite variable rates for *S. aureus*; (9.1%) in Indonesia (Lestari *et al.,* 2008), (14.8%) in Pakistan (Anwar *et al.,* 2004), (23.4%) in Malaysia (Choi *et al.,* 2006), (24.1%) in Taiwan (Lu *et al.,* 2005), while in China the prevalence of nasal *S. aureus* was (23.1%) (Ma *et al.,* 2011). Also, partially disagree with results of other reports from Africa which showed rates for *S. aureus*; (13.0%) in Tunisia (Ben Slama *et al.,* 2011), and (18.3%) in Kenya (Omuse *et al.,* 2012).

Culture on Nutrient agar: In this study, the morphological characteristics of *S. aureus*, *S. lugdunensis*, *S. epidermidis* and *S. hominis* isolates on the nutrient agar plates were matching with the morphological characteristics of *Staphylococci* obtained by (Blair, 1985) which referred that the colonies of *Staphylococci*, on the routinely used nutrient agar (NA), are white or nearly white; since it is probable that they are actually pigmented strains, the presence of colour may be determined by scraping up some of the growth with a loop and comparing it with a white background, the colonies are round, raised, opaque, smooth and glistening, and exhibit characteristic pigmentation, which ranges from deep gold to yellow, cream or white. In addition, the results of this study in agreement with the findings obtained by other study showed that the bacterial culture carried out by conventional methods on routine media such as nutrient agar, and also referred that the Staphylococci grow readily on many types of media, active metabolically, fermenting carbohydrates and producing pigments that vary from white to yellow; the colonies on solid media are round, smooth, raised and glistening (Brooks *et al.,* 2007).

Culture on Blood agar: In current study, the morphological characteristics of *S. aureus*, *S. lugdunensis*, *S. epidermidis* and *S. hominis* isolates on the blood agar plates were matching with the morphological characteristics of *Staphylococci* obtained from the study conducted by (Mamza *et al*., 2017), which showed that the colonies of *Staphylococci* on the blood agar were round, white-gray, or golden yellow colonies, and with the study conducted by (Ochei and Kolhatkar, 2008), which referred that the staphylococci colonies on most media are round, smooth, raised and measuring 1-2 mm in diameter. Also, the *S. aureus*, *S. lugdunensis*, *S. epidermidis* and *S. hominis* isolates on the blood agar plates showed different types of hemolysis, and this results match with findings of (Blair, 1985) study that explained when the freshly staphylococci isolated and grown on blood agar, many strains exhibit a zone of hemolysis around the colonies; some strains are non-haemolytic, the staphylococci produce several haemolysins, includes α-haemolysin and β-haemolysin, the α-haemolysin is active against rabbit and sheep erythrocytes, but human red blood cells are relatively resistant to its action. On blood-agar plates a clear zone of haemolysis is observed after incubation at 37°C for 18-24 hours. When freshly isolated from lesions in man, many strains produce α-haemolysin, and this property is regarded by some as presumptive evidence of the pathogenicity of staphylococci, whereas the β-haemolysin produces lysis of sheep, bovine and human, but not of rabbit erythrocytes. On blood-agar plates a small central zone of clear haemolysis, surrounded by a wide zone of darkening or of partial haemolysis, is characteristic of, β-haemolysin; the degree is increased when the plate is subsequently held under refrigeration.

Culture on mannitol salt agar: In this study, the morphological characteristics of *S. aureus*, *S. lugdunensis*, *S. epidermidis* and *S. hominis* isolates on the mannitol salt agar plates were matching with the morphological characteristics of *Staphylococci* obtained from the studies conducted by (Bannerman, 2003; Mamza *et al*., 2017), explained that the mannitol salt agar is a medium for the isolation of staphylococci, and about (84%) of the staphylococci isolates in them study when grown on it, produced colonies which were smooth, raised, and glistening, with characteristic gray, pink, and pale or deep yellow coloration or pigments, other studies showed that the *S. aureus* but no other staphylococci ferment mannitol, and usually form gray to deep golden yellow colonies on mannitol salt agar (Brooks *et al.,* 2007), the isolates which demonstrated characteristic appearance of *S. aureus* were found to be Gram-positive, appearing in clusters, singles, pairs, tetrads or chains (Ochei and Kolhatkar, 2008). Also, other studies referred that the staphylococci have the unique ability of growing on a high salt containing media (Koch, 1942), and isolation of coagulase-positive staphylococci using the phenol red mannitol agar supplemented with (7.5%) NaCl (Chapman, 1945). Mannitol is the fermentable carbohydrate, fermentation of which leads to acid production, detected by phenol red indicator. *S. aureus* ferment mannitol and produce yellow coloured colonies surrounded by yellow zones (Hitchins *et al*., 1995; Murray *et al*., 2003), the resulting mannitol salt agar is recommended for the isolation of coagulase positive staphylococci from cosmetics, milk, food and other specimens (Davis, 1959; Silverton and Anderson, 1961).

Vitek 2 system: The using of VITEK 2 system in present study to diagnosis the *Staphylococcus* species was supported by the findings of O'Hara and Miller, (2003) study that referred because relatively some of the phenotypic identification procedures are based on colorimetric or pH-based changes and usually require 18 to 24 h to identify organisms and some of them are based on changes in preformed enzymes, shortening the time necessary to make the identification to 2-4 hours. The Bact/Alert instrument is designed to decrease the turnaround time for the identification of bacterial isolates. In independent study, exhibited that the methicillin-resistant strains of *Staphylococcus aureus* (MRSA) are important pathogens causing nosocomial and community-acquired infections, some coagulase-negative staphylococci (CNS), such as *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, are also frequently methicillin- and multidrug-resistant and cause bloodstream and device-related infections, invasive isolates of staphylococci should therefore be identified to the species level to determine their

clinical relevance and to monitor their epidemiology, so in total, 318 isolates of *Staphylococcus* spp. Comprising 197 MRSA isolates from the National MRSA Surveillance Programme, and 121 clinically significant *Staphylococcus* spp. isolates from blood culture (76 *S. aureus*, 35 *S. epidermidis*, 3 *S. haemolyticus*, 3 *S. lugdunensis*, 2 *S. hominis*, 1 *S. schleiferi* and 1 *S. intermedius* obtained during 1998. 34 (45%) *S. aureus* and 29 (83%) *S. epidermidis* were resistant to oxacillin (Kloos and Bannerman, 1994). The results of Vitek 2 system of present study go together with results of the study that mentioned above which showed the Vitek 2 exhibited excellent accuracy for detection of *S. aureus* and coagulase negative staphylococci (CNS) isolates that are encountered less frequently in clinical specimens, including *S. lugdunensis, S. haemolyticus* and *S. hominis* through the identification correctly 111(92%) of 121 bloodstream isolates of staphylococci: 74 (97%) of 76 *S. aureus*, 31 (89%) of 35 *S. epidermidis*, and 6 of 10 other CNS species. In addition, different studies referred that the Vitek 2 system showed acceptable accuracy for automated identification with staphylococci isolates, the ability to offer rapid identification for various bacterial species makes this system attractive to clinical laboratories, although the laboratory workflow meant that the results of susceptibility tests were often not available until the next working day (Maes *et al*., 1997; Couto *et al*., 2001; Edwards *et al*., 2001). Other studies showed that the VITEK 2 system is capable of identifying *S. lugdunensis*, which is strongly recommended because it can produce infections that are just as severe as those caused by *S. aureus*. In clinical laboratories that use screening tests alone, *S. lugdunensis* can be misidentified as *S. aureus* or other coagulase-negative staphylococci (Schnitzler *et al*., 1998). Whereas in the older VITEK system, whose database did not include this species, (79%) of *S. lugdunensis* isolates were misidentified and the remaining (21%) were unidentified, the VITEK 2 system correctly identified all the *S. lugdunensis* isolates tested (Bannerman *et al*., 1993). In conclusion, different studies referred that the accurate identification of staphylococcal isolates is crucial for the correct management of staphylococcal infections, but it is also essential for a better understanding of the pathophysiological factors affecting the clinical outcome and for epidemiological surveillance (Archer, 1995), thus; the clinical laboratories will have an increasing need for rapid and reliable methods of identifying the causative organisms at least at the species level, on the whole, the VITEK 2 system provided accurate and reliable results for the staphylococcal isolates, with considerable savings in terms of time and work. This experience suggests that the system can be used in clinical laboratories for routine identification of staphylococci isolates responsible for different infections (Benjamin *et al.,* 2001).

DNA extraction: The result of current study showed the important use of commercial kits such as genomic DNA extraction kit as a rapid extraction method for the genomic DNA comparing with conventional DNA extraction methods such as alkaline lysis, boiling and salting out methods, and this finding agree with finding of (Mahnaz *et al.,* 2012) that they referred to interest use of the genomic DNA extraction kit (AccuPrep Bioneer Corporation) as an effective and rapid method for extracting the DNA. This eliminated the use of phenol-chloroform which is a cumbersome and tedious step of other PCR methods, resulting in significant improvements in the processing speed.

Detection of *nuc* gene for *S. aureus*, *S. lugdunensis*, *S. epidermidis* and *S. hominis* isolates by multiplex PCR: Many studies referred that the Staphylococci are identified by phenotypic analysis in many clinical laboratories. Although many phenotypic identification methods are commercially available, diagnostic accuracy has been reported to be 36.7 to 93.6% (Carretto *et al*., 2005; Heikens *et al*., 2005; Kim *et al*., 2008), this inaccuracy is problematic in clinical practice (Layer *et al*., 2006). Other studies revealed that because coagulase negative Staphylococci (CNS) are closely related to prosthetic device infections, CNS isolated from blood cultures of previously healthy individuals may be regarded as contaminants, however *S. lugdunensis* can cause native valve endocarditis, and community-acquired *S. lugdunensis* bacteremia has been associated with endocarditis and failure to identify *S. lugdunensis* might lead to delayed or inadequate treatment with an increase in morbidity and mortality (Choi *et al*., 2010; Zinkernagel *et al*., 2008). Little is known about the ecology of CNS despite commensal bacteria. It is thought that *S. capitis* is seen mostly on the head (Rogers *et al*., 2009), however isolates have been identified not phenotypically but genotypically also, and thus the genetic analysis is needed for definitive species identification (von Eif *et al*., 2002). The using of multiplex-PCR for species identification of staphylococci isolates of present study match with findings of the study carried out by (Baba *et al*., 2009) exhibited that the *nuc* gene was generally found in staphylococci except the *S. sciuri* group, and referred to develop a multiplex-PCR method for species identification of staphylococci based on nucleotide sequences of the *nuc* gene which showed that the *nuc* gene was present in 24 staphylococcal strains but absent in the *S. sciuri* group, the multiplex-PCR has been proved to be a rapid and accurate method for species identification of human-associated staphylococci.

Acknowledgement

I would like to acknowledge Prof. Dr. Abdul-Hussein Al-Faisal, Dean of Institute of Genetic Engineering and Biotechnology for Post Graduate Study, Assistant lecturer Miss. Noor E. Al-Bayati and Mrs. Zainab H. Al-Husseiny.

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