Rapid Molecular Characterization of Virulence Pattern in Clinical Origins Methicillin Resistant Staphylococcus Aureus Including Cytotoxin Panton Valentine Leukocidin

Halah J. Kadhim, Siham Hamel Mohaisen and Abdulameer M. Ghareeb
Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Baghdad, Iraq.
Corresponding author: halha81071@gmail.com

Abstract

Panton-Valentine leukocidin (luk-pv) producing Methicillin Resistant Staphylococcus aureus (MRSA) is a serious health threatening pathogen causes a skin and soft tissue infections. Accurate and rapid diagnosis of this pathogen determination the antibiotics resistance and virulence patterns is contributed significantly in effective treatment and improves the proceedings of infection control. In this study, single reaction of polymerase chain reaction technique applied to characterize the MRSA pathogen and determine the prevalence of its most important virulence genes including: luk-pv, mecA and nuc genes encoded for Panton-Valentine leukocidin, penicillin binding protein and thermo stable nucleasase respectively, in addition to species-specific detection using 16S rRNA. Successful reactions were gathered in one multiplex reaction. In total 155 of different clinical samples were collected from main hospitals in Baghdad. The results showed variant profile of resistance against 18 antibiotics; complete resistance (100%) toward Methicillin and Oxacilline. High resistance to Ceftazidime (92.85%), Ampicillin (91%), Piperacillin (85.7%) and penicillin G (77%). Moderate resistance was to Tetracycline (67%), Cefotaxime (58.57%) and Clindamycin (34.28%). While low resistance was towards Tobramycin (14.28%), Vancomycin (17%), Gentamicin (18.57%), Levofloxacin (55.7%) respectively. No clear correlation was observed between existence of pvl nuc and antibiotic pattern. Obviously, the luk-pv prevalence is significantly high in Baghdad. Optimization the monoplex PCR reactions in union multiplex reaction is succeed. Therefore, such sensitive and specific with no false positive result is reliable assay can be adapts for routine use in a diagnosis of MRSA pathogen.

Keywords: Multiplex PCR; Panton Valentine Leucocidin; MRSA; Baghdad

Introduction

Staphylococcus aureus is recognized as one of the most important bacterial pathogens causing a wide range of infections (Talebi-Satlu et al., 2012). Specific group of S. aureus carrying staphyloccocal cassette chromosome mec (SCCmec) characterized as Methicillin resistance Staphylococcus aureus (MRSA) has identified as a significant public health concern causing morbidity, mortality and long hospitalization (Talebi-Satlu et al., 2012). Virulence factors have essential impact on MRSA pathogenicity and contributed in its spread. Panton-Valentine-Leucocidin (luk-pv) is one of these factors as a powerful cytoxin forming pores for human mononuclear cells, disruption of leukocyte membranes and can cause cell death by apoptosis (Abdulrazaq et al., 2014). The genetic material of bacteriophage has great contribution in the production of luk-pv cytoxin (Shrestha et al., 2014). MRSA strains those producing luk-pv are more virulent and highly transmissible strains than luk-pv negative. luk-pv cytoxin encoded by luk-pv genes (lukS-pv and lukF-pv ) that encodes luk-pv composed of two exoprotein subunits which are transcribed together and secreted separately to form a complete heptavalent leukocidin (Kaneko & Kamoio, 2004; Prevost et al., 1995). MRSA can be classified into community acquired (CA-MRSA) and hospital acquired (HA-MRSA) infections, which CA-MRSA show more-diverse clonal groups than HA-MRSA and belong to SCCmec type IV, V, or VI DNA element (Kloos & Wolfshohl, 1982; Fritz et al., 2008). The pvI gene region characterizes as a significant virulence factor and conservative genetic marker of CA-MRSA strains (Monday & Bohach, 1999). The reports had shown a high prevalence proportion 77% of luk-pv in CA-MRSA while it presents only 4% of HA-MRSA isolates (Yoong and Torres, 2013). The luk-pv gene can spread among strains through bacteriophage and plasmids transmission results gradually increased prevalence of luk-pv carrying strains (Bush et al., 1995: Issa, 2019). Limited records on luk-pv prevalence has been reported in Baghdad the capital of Iraq. Therefore, applicable molecular diagnosis method was used to determine the luk-pv prevalence in addition to the main virulence factors and antibiotic resistance profile in clinical strains of MRSA.

Materials and Methods

Clinical samples collection: A total of 155 samples were collected from different clinical specimens from hospitalized patients attended specific hospitals (26 % of the total was from Al-Kindi, 24% from Al -Zaafaranayiah General Hospital, 30 % from the Medical city, 20 % from Al-Karkh hospital) in Baghdad during the period from December 2018 to the end of April 2019. Seventy isolates were obtained from the following sources: C.S.F (n=2), ear (n= 7), wounds (n=13), H.V.S (n=5), burn. (n=17), Abscess. (n=10), Urine (n=13) and Blood (n=3).The isolates were identified by means of routine tests: colony morphology and pigment formation on selective medium (mannitol salt agar), catalase test, oxidase reaction, coagulate test. Identification was confirmed via Api staph, VITEK 2(bioMerieux).

Antimicrobial susceptibility: The Antibiotic susceptibility testing was carried out for all the isolates on Mueller-Hinton method using disk diffusion method (CLSI, 2018) to measure zones of inhibition against slandered concentrations for the following antibiotics:
B: PCR amplification: Phenotypically characterized S. aureus isolates were subjected for molecular identification through single PCR amplification of species-specific 16SrRNA gene with expected size 288bp. Positive strains were subjected to the detection of mecA, nuc and luk-pv genes with expected size of amplified regions: 147bp, 270bp and 433bp, respectively. All reaction mixtures were set up 25 µl reaction mixtures as follows: 12.5 µl ready to use PCR master mix mixture (Promega) containing approximately 10 ng of template DNA and 1 µl (10 pmol) of each primer. The rest volume completed with deionizer water. The mixtures were subjected to the following thermal cycling parameters in a Thermocycler (Applied BioSystem): Initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification with 94°C for 45 sec, annealing at 50°C, for 1Min for the following genes 16S rRNA, mecA, nuc and 57 luk-pv respectively, extension at 72°C for 1 Min, and a final extension at 72°C for 6 min. The PCR products were analyzed on a 2% agarose gel. Combinations of the four target genes in one reaction was designed and performed as Multiplex PCR. Annealing temperatures were optimized using 50°C.

Results and Discussion

1. Isolation: A total number of 155 clinical samples (swabs) were collected from various patients and hospital staff in different units in four hospitals in Baghdad. The results included 70 positive samples of swabs diagnosed as S. aureus from the following different sources: 17 burns, 13 Wounds, 14 urine, 9 abscess and boils, 7 ear, and 5 swabs of vaginal (H.V.S), 3 Blood and 2 CSF. Diagnosed isolates was determined according to conventional cultural and microscopic characteristics was grape-like clusters of blue colour under microscopic examination (Subhankari, 2011; Benson, 2002). On mannitol salt agar yellow colony indicated a mannitol salt fermenting (Jayasundara, 2014; Bush & Jacoby, 2010). Also Identification of S. aureus by API Staph System (Kloos, & Wolfshohl, 1982) and Vitek 2 automated system (bioMérieux, France). (Ligozzi,2002). Confirmed isolates identified as SA# for further experiments.

2. Antibiotic Sensitivity: The resistance pattern to the 18 antimicrobials tested is shown in table 2 according to Clinical Laboratory Standards Institute (CLSI) guidelines. According to the results, isolates had the lowest rate resistant to Ciprofloxacin (4%). This shows that this antibiotic the last choice of therapy for these infections. According to the results, isolates had the lowest rate resistant to Ciprofloxacin, Cephalosporin antibiotics are even more resistant to β-lactamases, although some β-lactamases have an increased affinity for cephalosporins (Simon and Sanjeev, 2007). Developed the second generation of penicillins to counteract the resistance of antibiotics, which includes Methicillin. Oxacillin, a synthetic semi-synthetic penicillin resistant to the resistance of antibiotics, which includes Methicillin. Oxacilline, a synthetic semi-synthetic penicillin resistant to Cephalosporin antibiotics are even more resistant to β-lactamases, although some β-lactamases have an increased affinity for cephalosporins (Simon and Sanjeev, 2007). Developed the second generation of penicillins to counteract the resistance of antibiotics, which includes Methicillin. Oxacillin, a synthetic semi-synthetic penicillin resistant to the enzyme Pencillinase (Al-Taai et al., 2017). The MRSA prevalence was 100%. Antimicrobial susceptibility testing revealed a high resistance rate (91%, 85% 92%) towards Ampicillin, Piperacillin and Cefazidime respectively. Study conducted by researcher indicated that the rate of isolation resistance clinical S. aureus of Ciprofloxacin was 23% which is unlike the current study (Bush et al., 1995). Cephalosporin antibiotics are even more resistant to β-lactamases, although some β-lactamases have an increased affinity for cephalosporins (Issa, 2019). Results showed that the most effective antagonists against isolated clinical MRSA isolates. Results showed that the most effective antagonists against isolated clinical MRSA isolates.

Table 1 : Primers sequences used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5-3)</th>
<th>Product length (bp=base pairs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SrRNA</td>
<td>F-GTACCTGATGAGTTATCC</td>
<td>228</td>
<td>(Monday &amp; Bohach,1999)</td>
</tr>
<tr>
<td></td>
<td>R-CGGACATCATGCTTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mecA</td>
<td>F-GTAAAGATATACCAAGTGATT</td>
<td>147</td>
<td>Zhang et al.,2005</td>
</tr>
<tr>
<td></td>
<td>R-AGCTATAGATGATAAGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nuc</td>
<td>F-GCGATTGATGGTGATACGGTT</td>
<td>270</td>
<td>Brakstad et al.,1992</td>
</tr>
<tr>
<td></td>
<td>R-AGCCAGCTGGCAGAATCAAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>luk-pv</td>
<td>F-ATCATAGGTGAAATGTTGACATGATCCA</td>
<td>433</td>
<td>McClure et al, 2006</td>
</tr>
<tr>
<td></td>
<td>R-GCATCAAGTGTATGGATAGCAAAGC</td>
<td></td>
<td></td>
</tr>
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F, forward; R, reverse
our results are compatible with those obtained by Researcher (Brady et al., 2007) as they observed that all isolates were resistant to Oxacillin and Methicillin and other β-lactam antibiotics,

While Al-Hossainy (2007), showed that VRSA were 20% among S. aureus, the result is close to our results. This results disagreed with finding of Al-Geobory (2011) in that the rate of resistant to vancomycin was 2.27%. Where the VRSA isolate among S. aureus is isolates 4 out of 50 (8%) (Mohammed, 2011).

3. DNA extraction: Total DNA was extracted from 70 S. aureus isolates using genomic DNA Extraction Kit (Geneaid). Extracted DNA was confirmed as bands by gel electrophoresis as shown in figure (1). DNA concentration and purity were measured by Nanodrop spectrophotometer, all the isolates had DNA concentration between (50-80 ng/μl) with purity about 1,7-2.

4. Molecular identification: Phenotypically positive isolates of S. aureus were confirmed using the specific initiator of the 16S rRNA gene by PCR using thermal cycler. The results showed all bacterial isolates belong to S. aureus (figure 2-A). The 16S rRNA PCR assay has been shown to be able to successfully identify multiple types of bacteria in multiple sample types Matsuda et al., 2007. According to study conducted by Al-Alak and Qassim (2016), the detection of 16S rRNA by PCR is rapid and reliable method as they observed that using 16s rRNA confirmed all the 126 staphylococcal isolates as S. aureus.

Use as Khudhr (2016); El-Hadedy &El-Nour, (2012) PCR carried out using 16s rRNA gene as a specialist. for S. aureus. In this method for identifying and confirmed all the staphylococcal isolates as S. aureus.

Methicillin resistant phenotypic identification of the S. aureus isolates was confirmed by the PCR detection of the mecA gene. The results of the detection of mecA 70/70 gene (100%) coded for anti-methicillin resistance gave a positive result for the gene as shown in figure (2-B), AL-Hasnawi, et al. (2013) found that the proportion of mecA (100%) was an approach to the study, Researchers Ghaznawi-Rad and Ekrami, (2018) also reported the rate of clinical MRSA isolates That gave a positive result for the mecA gene was 100% as well, is a similar result with the current results, While In a study conducted by the researcher Rahama et al. (2017) found that 85.37% of mecA gene is not similar to the current study.

The results of the detection of the nuc 61/70 (85.70%) gene encoded for thermal cores gave a positive result of the genetic (figure 2-C). Existence of nuc gene in MRSA strains is 100% (Alagely, 2016; Brakstad et al., 1992) Found all isolates give positive result to nuc gene. However, there is study showed MRSA strains with negative nuc explaining this due to mutation in primer-annealing site or partial deletion in the gene. (Klaassen et al., 2003).

In order to detect the presence of luk-pv gene (luk-pv) and determination the prevalence of it among MRSA clinical isolates, single-plex polymerase chain reaction for each DNA extracted sample have been used. The results of the detection of the luk-pv 64/70 (91.40 %) gene encoded for Leukocidin gave a positive result (figure 2-D). The found researcher
Kareem, (2016) in Baghdad. Also, the results have been revealed (6.55%) of MRSA isolates harboring the pvl gene; that the percentage of pvl gene which is a little percentage compared to the current study.

In found Kandala et al. (2017). The ratio (luk-pv) is 53.48% which is moderate. Al-Hassnawi, et al., 2013 found that the percentage of luk-pv (79%) gave a positive result which is comparable to the current results. The researcher Rahama et al. (2017) carries the gene luk-pv 31.71% which is a small result for the study. For this study, the luk-pv gene was more prevalent. Where luk-pv was the gene carried by methicillin-resistant Staphylococcus aureus. It is a convergent result with Aghmiyuni et al. (2016). The study showed a relatively high prevalence of luk-pv in Staphylococcus aureus in India city.

All PCR products have been confirmed by analysis of the bands on gel electrophoresis and by comparing their molecular weight with 100 bp DNA Ladder.

In this study, rapid and easy PCR assay was designated to diagnosis MRSA strains those carrying virulence factors through combine the four single reactions of PCR in union multiplex assay. The Optimization is depends on select the optimum annealing temperature (55°C) using gradient option thermal cycler. Similar thought was conducted by Strommenger et al., (2008).

Developed a new multiplex PCR assay for detection of Panton-Valentine leukocidin virulence genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci, this assay is simple, rapid, and accurate and offers the potential for prompt detection of newly emerging CA-MRSA (McClure et al., 2006).

The significant spread of MRSA and the introduction of these highly pathogenic virulent isolates represent increasing health threats. Thus, sensitive and specific with no false positive result is reliable assay can be adapts for routine use in a diagnosis of MRSA pathogen might be good tool to accurate diagnosis and determine the correct treatment resulted in good management.
This study demonstrates the luk-pv gene is highly prevalent (91%) among MRSA strains in Baghdad governorate patients spread. Multiplex PCR is highly recommended to be used to detect genes as one approach to identifying MRSA in routine diagnostic laboratory for its rapid and precise diagnostic properties suggested it can be replaced to traditionally assays. A lower prevalence of luk-pv has been reported from other parts of world (5% in France, 4.9% in UK, 8.1% in Saudi Arabia, and 14.3% in Bangladesh) (Lina et al., 1999; Holmes et al., 2005; Afroz et al., 2008) reflecting the significant variation in prevalence of luk-pv among geographical areas and communities. Kaur et al., from India, have reported overall 62.85% prevalence of luk-pv among MRSA and MSSA (MRSA: 85.1% and MSSA: 48.8%), Johnsson et al. (2004) detected luk-pv gene in one isolate (1%) from 65 patients with S. aureus bacteraemia, in two (2.19%) isolates from 91 patients with cutaneous infections, and in four (7.27%) isolates from 55 patients with respiratory tract infections.

Reference
Al-Geobory, H.A. (2011). Comparative study between Methicillin resistant Staphylococcus aureus (MRSA) and Methicillin sensitive Staphylococcus aureus (MSSA), and detect the antimicrobial effects of some plant extracts on them. M.Sc. thesis. College of Science. Baghdad University
from Egyptian food by conventional and molecular methods, Journal of Genetic and Biotechnology, 129-135.


aureus (MRSA) infections presenting in the community. JAC., 61: 976-994.


