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SPA-TYPING OF *STAPHYLOCOCCUS AUREUS* ISOLATED IN ASTANA

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ABSTRACT

Staphylococcus aureus **colonization presents as a wide range of clinical infections and can lead to severe complications including death in human subjects. Currently, routine monitoring practices include methods of genetic fingerprinting for tracing outbreaks, as well as for global epidemiological studies. The use of rapid PCR-based methods including SPA-typing allow for classification of isolates with high virulence and drug resistance. The purpose of this study was to characterise isolates from Astana city hospital inpatients in 2017. A total of 153 isolates were collected. SPA-type could not be identified for seven samples, whereas as many as 61 SPA-types were found for the remaining 146 isolates. The most prevalent SPA-types were: t521 (10.6%), t267 (9.8%), t002 (6.5%), t024 (5.9%) and t091 (5.9%). Notably, low antibiotic resistance was found in 3.2% of MRSA isolates and in 7.8% of MSSA-MDR isolates. In 153 strains of *S. aureus*, a high percentage of resistance was found to trimethoprim (96.7%) and penicillin (84.3%). Erythromycin, clindamycin and tetracycline resistance were recorded in 6.5% of the isolates, and chloramphenicol, oxacillin, cefoxitin, doxycycline, ciprofloxacin and gentamicin resistance did not exceed 4%. All isolates were sensitive to vancomycin, linezolid and rifampicin. Regular monitoring can improve treatment program effectiveness and help to control the circulation of antibiotic resistant strains.**

Key words: *Staphylococcus aureus* antibiotic resistance, SPA-typing *staphylococcus aureus*, bacterial analysis in hospitalized patients

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INTRODUCTION

Staphylococcus aureus is an anaerobic gram-positive microorganism with spherical (cocci) cells, which may cause a range of distresses in humans and animals [1]. In addition to a number of skin infections *Staphylococcus aureus* can cause more dangerous diseases such as pneumonia, meningitis, sepsis, etc., and it is one of the main causes of hospital infections [2]. Because of ability the microorganism to adapt to various environmental conditions, *S. aureus* remains among pathogens which pose great concerns among researchers [3, 4]. Mortality from staphylococcal bacteremia remains approximately high (up to 20-40%) despite an availability of antimicrobial drugs [5]. At the same time, approximately 30% of population is asymptomatic carriers of *S. aureus*, and apart from being at a higher risk of infection, these people are the source of *S. aureus* strains [6, 7, 8].

Staphylococcus aureus strains differ by resistance to majority of existing classes of antibiotics. In early years of the penicillin era, the antibiotic resistance in *S. aureus* was a result of an acquisition of plasmids which carried genes of penicillinases (β-lactamases). The resistant strains produce the enzyme called β-lactamase which inactivate β-lactam ring in penicillin's chemical structure. Attempts were made to synthesize penicillin derivatives that are resistant to the inactivation by β-lactamases. This goal was achieved in 1959 with a synthesis of methicillin which have a phenolic group of benzylpenicillin substituted by methoxy groups. Methoxy groups produced steric hindrance around an amide bond in the β-lactam ring thus reducing its availability to hydrolisys by staphylococcal β-lactamases [9]. The new drug was introduced into medical practice, but as early as in 1960s the first methicillin-resistant strain of *Staphylococcus aureus* was discovered (*methicillin-resistant Staphylococcus aureus, MRSA*) [10]. Resistance to β-lactam antibiotics in the MRSA strains is mediated by a *mecA* gene which encodes a penicillin-binding protein 2a (PBP2a, or PBP2'). The *mecA* gene has a size of 2.1 kb and is found on a mobile genetic element known as a staphylococcal chromosomal cassette *mec* (*SCCmec*). The *SCCmec* element comprises a *mec* complex which in turn contains the *mecA* gene, regulatory genes and insertion sequences (*IS431, IS1182, IS1272*). The mobile element also includes either one (*ccrA*) or two (*ccrA* and *ccrB*) site-specific recombinases and 3 connecting regions [11].

In addition, *Staphylococcus aureus* is able to produce a wide range of virulence factors, including toxins. The most studied is the so called Panton and Valentine toxin, initially found in a strain which was isolated from a patient with chronic furunculosis [12]. This is gamma toxin capable of increasing permeability of membranes of monocytes and macrophages, and causing cell death [13].

A modern clinical laboratory practice usually includes a confirmation of a status of MRSA and determination of a phenotypic antibiotic resistance for isolates under consideration. Genetic methods of intraspecific typing are necessary for conducting of epidemiological analysis, determining a group or cluster to which the *Staphylococcus aureus* isolate belongs. A method of Multilocus Sequence Typing (MLST) has long been considered as a primary method for a designation of clonal lines (ST) and clonal complexes (CC) for *S. aureus*. However, new method of SPA-typing was recently introduced for these purposes. The SPA-typing is based on a sequencing of a single polymorphic locus: site X of the staphylococcal protein A gene. Protein A (SpA) -components of the cell wall of *S. aureus* closely interacting with the host immune system by inhibiting its effect on the cell. SpA contains an N-terminal signal sequence followed by five repeated domains (E, D, A, B, C) that bind IgG. IgG binds to the surface of bacterial cells in the wrong orientation, which leads to disruption of opsonization and phagocytosis. SpA is anchored to the surface of the bacterial cell through a carboxy terminal motif. The carboxy terminus of SpA contains variable numbers of a 24-bp repeat sequence known as the X region [14]. The method finds more and more proponents, because it is suitable for the analysis both local (within a same hospital) and global spread of *Staphylococcus aureus* [15]. Genetic typing of spa is a suitable technique for distinguishing between nosocomial and community acquired sources of prosthetic shunt graft infections [16].Clustering strains by geographic attachment provides valuable information for for epidemiological case tracing and allowed conclusions to be reached on the importance of newly emerging reservoirs [17].

Despite a danger to population health posed by *Staphylococcus aureus* in Kazakhstan, molecular genetic analysis is not sufficiently developed in the country, and still there is no literature published on a genetic diversity of the circulating strains. A purpose of this work is to analyze a distribution of the MRSA strains of *Staphylococcus aureus* and describe epidemiologically significant *SPA*-types of the isolates.

MATERIALS AND METHODS

Design of the experiment and isolates of *Staphylococcus aureus*

This study was conducted for 6 months, from February to July 2017. The study was approved by the local ethical committee of the infectious diseases hospital in the Astana city. For all hospitalized patients standard diagnostic procedures were carried out including isolation of the *S. aureus* cultures. A total of 153 isolates of *Staphylococcus aureus* were obtained. Primary identification of *S. aureus* was carried out by morphological and biochemical methods. A confirmation of initial species identification was carried out by a direct sequencing of the *16S rRNA* gene fragment using primers: 8f (5'–AGAGTTTGATCCTGGCTCAG-3) and 806R (5'-GGACTACCAGGGTATCTAAT-3') as described in [18].

Sensitivity to the antibacterial drugs

Determination of sensitivity of the *S. aureus* isolates to antibiotics was performed by disc-diffusion method on the Mueller-Hinton agar according to the recommendations of CLSI (*Clinical and Laboratory Standards Institute*) [19]. Used discs (HiMedia) included the following list of 14 antibiotics: Penicillin (PG 10 μg), Oxacillin, Cefoxitin, Erythromycin, Clindamycin, Trimethoprim, Doxycycline, Vancomycin, Tetracycline, Rifampicin, Linezolid, Ciprofloxacin, Gentamicin, Chloramphenicol. Results of the test were taken after 24 hours of incubation at 37°C. Strains which were resistant to ≥1 drug in ≥3 discrete antimicrobial categories were referred to as methicillin-susceptible multidrug resistant (MSSA-MDR) strains [20].

DNA extraction, MRSA screening and PVL

DNA isolation was performed using a Qiagen QIAamp DNA Mini Kit in accordance to the manufacturer's instructions. A concentration of DNA was determined by a spectrophotometry at 260 nm. The MRSA phenotype identification was performed by a detection of the methicillin resistance encoded in genes *mecA*, *mecC* using *16S rRNA* and thermostable nuclease (*nuc*) as controls. Detection of the leukocidin toxin (Panton-Valentine toxin, PVL) was done as previously described by Stegger et al [21, 22]. An analysis of PCR products was performed by electrophoretic separation of the DNA fragments in a 1.5% agarose gel, in a presence of ethidium bromide.

Spa typing

Amplification of the *spa* gene was performed using primers and reaction conditions described by *Harmsen D.* [23]. Purification of the PCR products was performed by enzymatic method using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) [24]. Sequencing reactions were carried out using *BigDye Terminator v3.1 Cycle Sequencing Kit* (*Applide Biosystems*) according to the manufacturer's instructions, followed by separation of the products of the sequencing reactions using an automatic genetic analyzer *3730xl DNA Analyzer* (*Applide Biosystems*). Numerical *SPA*-repeat codes and *SPA*-types were determined in accordance with the nomenclature of *Ridom SpaServer* (http://spa.ridom.de) using a plug-in for the *SPA* typing *BioNumercs V 6.6* (*Applied Maths, Belgium*).

RESULTS

Characteristics of the study population

Isolates of *S. aureus* were obtained from 153 patients undergoing inpatient treatment at the city hospital in the Astana city from February to July 2017. Total number of participants in this study is 153, a ratio between men and women being 60:93. The patients were diagnosed with lacunar tonsillitis or occasionally misdiagnosed with an acute respiratory virus infection (ARVI).

In women group, there was a nearly equal partitioning between the diagnoses. Among men, the diagnosis of lacunar angina was threefold more common than the ARVI.

Table 1. Characteristics a group of patients

Sex	Number of *S. aureus* isolates (%)	Age, years (mean age)	Diagnosis	
			lacunar tonsillitis	ARVI
Women	93 (60,8%)	17-76 (29 ± P=0.7)	44 (47,3%)	49 (52,7%)
Men	60 (39,2%)	16-44 (25 ± P=0.7)	46 (76,7%)	14 (23,3%)

MSSA-MDR, MRSA, sensitivity to antibiotics and detection of PVL in the circulating *S. aureus* isolates

Five of the 153 *S. aureus* isolates were assigned to MRSA by the mecA sequencing, none of the isolates had mecC. Twelve isolates had resistance to more than one antibiotic belonging to three or more categories of the antibiotics, and these isolates were recorded as MSSA-MDR. From the 153 *S. aureus* strains, a high fraction of the resistant strains was found to Trimetoprim (96.7%) and Penicilin (84.3%). To Erytromycin, Clindamycin and Tetracycline, resistance was recorded in 6.5% of isolates. For Cloramphenicol, Oxacilline, Cefoxitin, Doxycililine, Ciprofloxacin, Gentamicin the resistance did not exceed 4%. All isolates were sensitive to Vancomycin, Linezolid and Rifampicin (Fig. 1).

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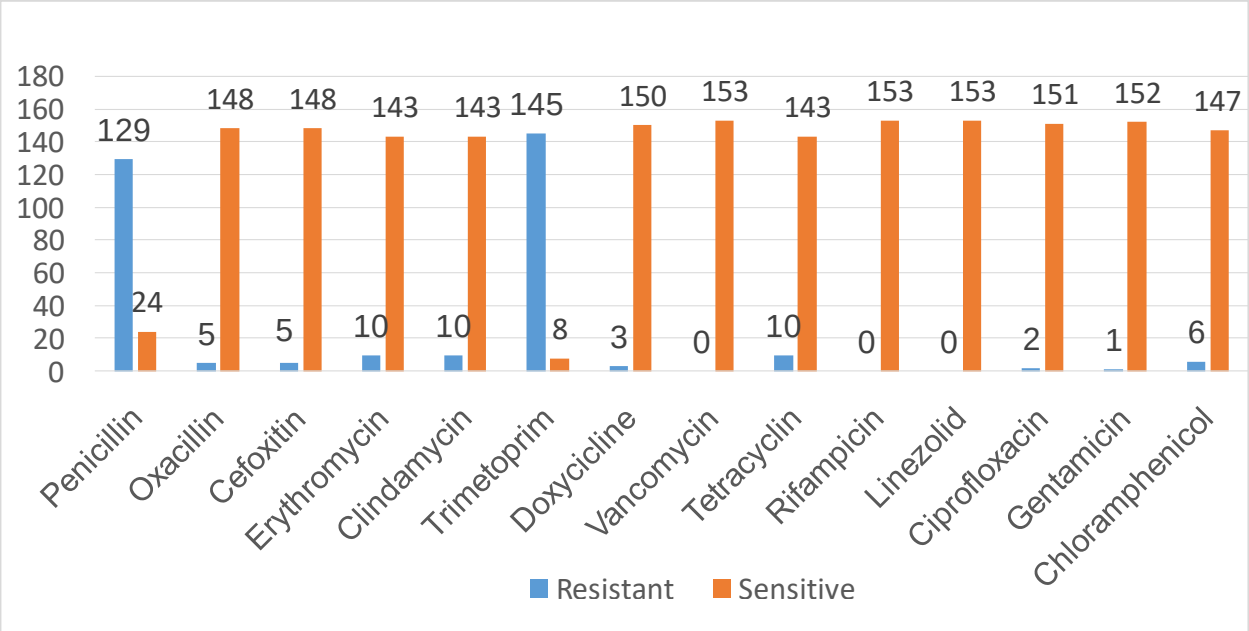

Fig. 1 – Sensitivity of isolates to antibiotics

Only 6 isolates (3.9%) were positive for PVL. Of these, one isolate was from a 76-year-old female patient with lacunar tonsillitis, and the isolate was MSSA-MDR.

SPA typing

SPA-typing was performed for all 153 isolates. For seven isolates, the used software algorithm failed to assign he SPA-type despite clear signals were observed in electrophoresis. In the other 146 isolates, 61 SPA-types were found (Fig. 2). The most common SPA-types among

the strains were: t521 (10.6% of isolates in n-16), t267 (9.8% of isolates in n-15), t002 (6.5% of isolates n-10), t024 and t091 (5.9% of isolates in n-9). The SPA-types t065, t156 and t223 included 5 isolates (3.3%); t164 and t359 included 4 isolates (2.6%). By three isolates belonged to 5 SPA-types (t050, t8053, t856, t8672 and t527), by two isolates belonged to 6 SPA-types (t015, t084, t127, t1376, t160 and t242). The remaining 41 isolates were divided into an equal number of SPA-types.

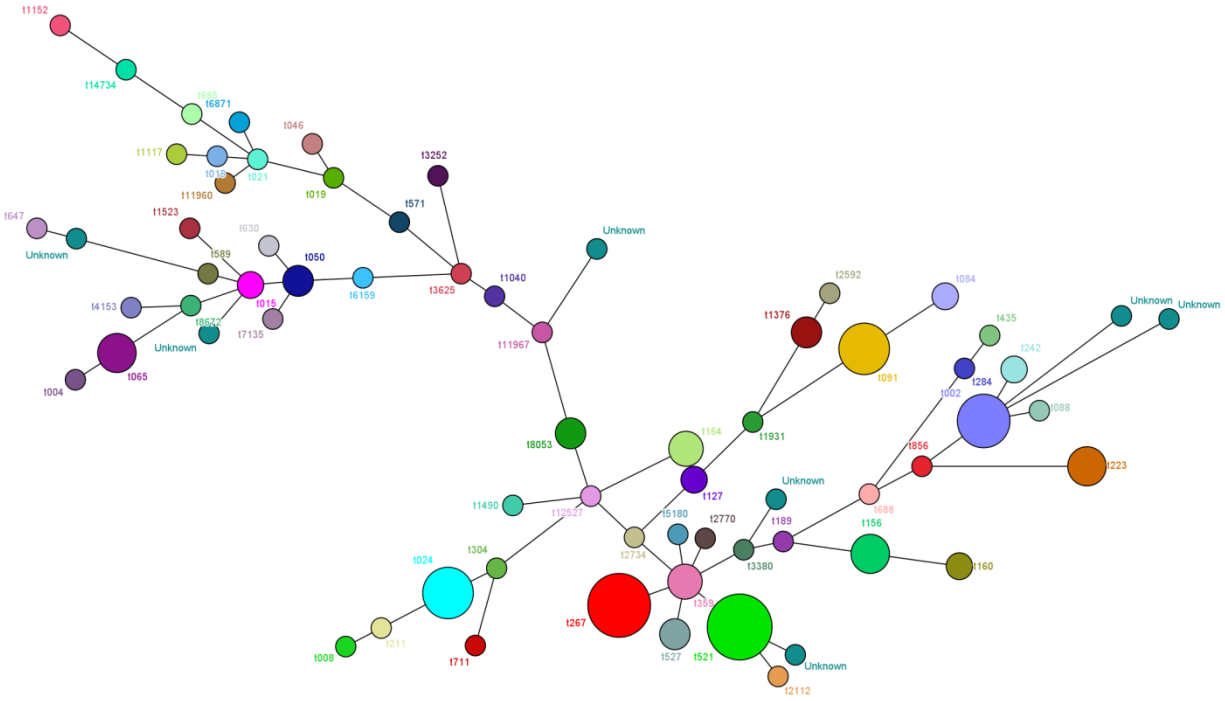

Fig. 2. Minimal spanning tree of *S. aureus* 153 isolates

An association between the SPA-type, drug resistance and coding of the toxine (PLV) was not revealed. Thus, the MRSA isolates are represented by the following 5 SPA-types: t002, t223, t2112, t024, t304. An isolate with a resistance to 10 out of 14 antibiotics (penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, trimetoprim/sulphamethoxazole, doxycicline, tetracycline, gentamicin, chloramphenicol) was found which belonged to a little-represented SPA-type t2112.

DISCUSSION

This study allows filling some gaps in knowledge about the SPA-types of *Staphylococcus aureus* circulating in Kazakhstan. In our study, the SPA-type was determined for 146 (from 153 studied) isolates, and 7 isolates were classified as "Unknown". Previous reports describe a detection of strains which could not be identified for the SPA-type [25]. This study shows existence of a high genetic diversity in *Staphylococcus aureus* strains. In our sampling frame, the most common SPA-types were t521 (10.6%), t267 (9.8%), t002 (6.5%), t024 and t091 (5.9%). One SPA-type T521 is poorly represented, as in the Ridom database only 0.04% of isolates have this SPA-type. Also the SPA-type t267 has 0.33% of the Ridom base isolates of which more than 48% are classified as MRSA. In our study, for the SPA-type t267 one isolate was classified as MRSA and two isolates as MSSA-MDR. The SPA-type t002 is the third most common type in the Ridom database. It is among the three most prevalent SPA-types in Asia, Europe and America, more than 85% of which are classified as MRSA [26, 27]. As is known the SPA-type t002 relates to ST5 and CC5 which are founders of major clonal groups, and contain the most

dangerous epidemic methicillin-resistant strains (MRSA) and MSSA [28, 29]. In our sample, none of the 10 isolates with SPA-type t002 was assigned to MRSA. Only the clonal types ST5, ST30 and ST45 are characterized by a presence of circulating strains with various types of SCCmec-cassettes. Genetic lines belonging to clones t002 (ST5) and t012 (ST30) often carry the Panton-Valentine leukocidin (PVL) gene, and this applies to both MRSA and MSSA. In our sample, in this group of isolates, PVL was not found. Fractions of the isolates with the SPA-types t024 or t127 were 5.9%. These types are commonly encountered worldwide, although their share in the Ridom database is 0.66 and 2.52% respectively. Two isolates of the SPA-type t024 were classified as MSSA-MDR and 1 isolate as MRSA. Among rarely represented entities attention should be drawn to the SPA-type t1376, both isolates of which possess PVL; and the SPA-type t2112 which one showed resistance to ten of 14 antibiotics. All isolates with PVL were found to be MSSA, although in the published literature in most cases PVL is associated with the MRSA isolates [30, 31].

Our study showed a low prevalence of MRSA (3.2%) and MSSA-MDR (7.8%) among clinical isolates of *S. aureus* in Astana. Due to a lack of published data to describe antibiotic resistance of *S. aureus* in Kazakhstan, we compared our results with those from China and Russia, because the countries are in a geographical proximity and there is a possibility of circulation of the strains across the borders. In various provinces of China, uneven levels of MRSA are reported. In Shanghai, 444 isolates (72.8%) were MRSA and 166 (27.2%) were MSSA [32]; in a sample from 12 provinces a prevalence of meticillin-resistant *S. aureus* (MRSA) was found to be 47.5% (112/236) [33]. In two Russian studies, prevalence of the MRSA strains varied from 18 to 48% [34, 35]. Due to limited sampling and homogeneity of observed pathologies (lacunar tonsillitis/ARVI), resistance to antibacterial drugs may be underestimated. Regular monitoring can improve effectiveness of treatment programs and slow down circulation of the resistant strains.

CONCLUSION

The *S. aureus* isolates circulating in Astana in patients with diagnoses of lacunar tonsillitis or ARVI are genetically heterogeneous by a polymorphic site X of a staphylococcal protein A gene. Low antibiotic resistance was noted: 3.2% for MRSA isolates, and 7.8% for MSSA-MDR isolates. The data can be used to create a system for a genetic surveillance of the *S. aureus* strains.

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