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MOLECULAR TYPING OF METHICILLIN-SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* (MSSA), ISOLATED FROM MONKEYS, BASED ON COAGULASE GENE POLYMORPHISM

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SUMMARY

Staphylococcus aureus is a very dangerous microorganism that causes more than 100 nosological forms of disease in humans and animals, including pneumonia, skin and soft tissue infections, food toxicoinfections, wound abscess, etc. Numerous studies on genotyping *Staphylococcus aureus*, isolated from humans, food and bovine mastitis have been carried out. The lack of information on the genotyping of these pathogens detected in monkeys living in captivity served as a stimulus to conduct a similar research, since staphylococcal infections in the primates are widespread. The present study is devoted to the study of the polymorphism of a variable region of the coagulase gene and to the typing of *Staphylococcus aureus* isolates from monkeys of different species kept at Adler monkey farm. 115 *Staphylococcus aureus* isolates were studied using phenotypic and molecular genetic methods. Genotyping was performed using PCR, real-time PCR and PCR with subsequent restriction fragment length polymorphism analysis (PCR-RFLP). A real-time PCR analysis allowed to classify all *Staphylococcus aureus* as methicillin-susceptible staphylococci (MSSA). After amplification of a variable region of the coagulase gene, 4 types of amplicons of 600, 700, 800, and 900 bp were generated. This data demonstrates structural differences of this gene in the studied isolates. The coagulase gene of 900 bp prevailed. The use of the *Cfo1* endonuclease allowed to identify 23 different restriction profiles of the *coa*-gene, but only three of them predominated. *Staphylococcus aureus* bacteria with seven types of coagulase gene were found only in the lungs of monkeys that died of pneumonia. The results obtained suggest that these isolates have tropism for lung tissue. Among *Staphylococcus aureus* isolated from pneumonia cases, isolates with three types of the *coa*-gene prevailed. *Staphylococcus aureus* of eleven types of coagulase gene can be attributed to the invasive isolates, since they were detected in the tissues of various organs. Staphylococcal infection in monkeys kept at the monkey farm is caused by genotypically heterogeneous population of *Staphylococcus aureus*.

Key words: monkeys, *Staphylococcus aureus*, coagulase gene, PCR-RFLP, coa type, RFLP-pattern.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a unique microorganism that is one of the critical opportunistic pathogens playing a significant role in infectious process emergence in animals. This microbe often causes pneumonia, flegmon, arthritis, suppurative inflammation of wounds, bone and joint lesions in domestic and wild animals (cats, dogs, cattle, sheep, horses, pigs, rats, seals, guinea pigs, etc.) [6, 14]. It is a causative agent of mastitis, postpartum endometritis in cows and sows [1, 2, 9, 15]. *S. aureus* bacterium was isolated from cats with conjunctivitis as a chlamydia-concurrent infection [4]. Moreover, *S. aureus* can asymptomatically colonize animals. Therewith, asymptomatic carriers normally act as the reservoirs of the infection agent during the *S. aureus*-associated outbreaks and are predisposing factor for the infection. Currently, much attention is paid to studies of methicillin-resistant *Staphylococcus aureus* (MRSA), detection rate of which has increased significantly for the recent ten years as well as to their transmission from people to animals and vice versa [6]. Literature data show that methicillin-susceptible *Staphylococcus aureus* (MSSA) is a potential reservoir for MRSA [7]. The significant virulent factor of *Staphylococcus aureus* is a coagulase. Its detection is used in laboratory practice as a criterion for differentiation of pathogenic *S. aureus* from other Staphylococcus species. Coagulase is an extracellular protein that is able to bind to prothrombin forming a complex called as staphylothrombin that stimulates clotting reaction of plasma by converting fibrinogen to fibrin [16]. Coagulase gene (*coa*) coding for coagulase protein is highly polymorphic due to

variable sequences having 81 base-pair tandem repeats at 3' terminus. There are many allelic forms of coagulase gene and each *S. aureus* strain produces one or several such forms [11]. Coagulase gene amplification depends on heterogeneity of the region comprising 81 base-pair tandem repeats at 3'-coding region of coagulase gene that varies in number of such tandem repeats as well as in position of restriction endonuclease sites in different isolates. Coagulase gene polymorphism is used as epidemiological marker; it is typed using primers homologous to conservative region within *coa*-gene [8]. Generated PCR products of some strains could be of different length due to number of repetitive sequences varying within *coa*-gene. Identification based on PCR-restriction fragment length polymorphism (RFLP) is considered to be simpler and more accurate method for *S. aureus* typing. DNA fragments separated with gel electrophoresis allows comparison of their lengths (RFLP patterns). PCR products of different strains show different RFLP patterns for coagulase gene. The said method of typing is very useful for testing strains and their genetic lines isolated from different sources for their origin as well as for epidemiological data generating [9, 11].

Currently, little attention is paid to *S. aureus* diagnosis and genetic investigation in animals and even much less in primates [3]. No molecular and genetic typing of *S. aureus* isolates recovered in captured monkeys is carried out abroad. Absence of data on *S. aureus* genetic diversity and molecular epidemiology in non-human primates have prompted the said study. Method for PCR-RFLP-based typing of coagulase gene for differentiation of *S. aureus* strains isolated from monkeys of various species was used.

The study was aimed at genotyping of *S. aureus* isolates recovered from monkeys by molecular typing of coagulase gene using PCR-RFLP method.

MATERIALS AND METHODS

S. aureus isolates (115 isolates) recovered from monkeys of 6 species living at Adler monkey farm: rhesus macaque ($n = 42$), crab-eating macaque ($n = 37$), pig-tailed monkey ($n = 2$), green monkey ($n = 5$), anubis baboon ($n = 5$), hamadryas baboon ($n = 24$). Therewith, 79 *S. aureus* isolates were recovered from lungs of animals died of pneumonia; 32 isolates were recovered from various organs such as kidney ($n = 5$), liver ($n = 10$), spleen ($n = 6$), lymph node ($n = 8$), uterus ($n = 1$), as well as from parenchymal fluid ($n = 2$). Four *S. aureus* isolates were recovered from live monkeys: pus from wounds ($n = 2$), eyes ($n = 1$), pharynx ($n = 1$).

Staphylococcus isolation and identification. Standard bacteriological testing including inoculation on selective and differential-diagnostic nutrient media (salt egg agar, 5% blood agar), microscoping of Gram-stained smear, tests of isolated cultures for their biochemical properties was carried out for staphylococci isolation. The materials were seeded onto nutrient media using common microbiological methods. Identification of staphylococci was performed based on their morphological, tinctorial, plasma-coagulating and biochemical properties and latex agglutination test results. Species identification was performed using commercial tests systems, Multimicrotests for biochemical identification of staphylococci (MMT C) (NPO Immunotex, Russia). Dryspot Staphytect Plus latex agglutination test kit (Oxoid, Great Britain) for *S. aureus* strain identification was used according its manufacture instruction for serological testing. 115 *S. aureus* strains selected for molecular genetic testing were stored at minus 20° C.

S. aureus genomic DNA extraction. Total *S. aureus* DNA extraction from bacterial suspensions (1.5×10^8 CFU/ml equal to 0.5 McFarland turbidity standard) prepared from day-old agar *S. aureus* cultures and suspended with 100 µl of NaCl using DNA-sorb-V reagent kit (OOO InterLabService, Russia) according to the manufacturer's instruction was performed.

S. aureus mecA gene detection with PCR. Real-time reaction amplification was carried out using commercial AmpliSense MRSA-screen titre-FL test system (OOO InterLabService, Russia) in RotorGene fluorescence-detecting thermocycler (USA) according to attached instruction. The commercial tests system allows *S. aureus* identification and detection of the genetic determinants (*mecA* gene) responsible for staphylococcus resistance to β-lactam antibiotics of penicillin group.

PCR detection of S. aureus coagulase gene. PCR amplification of 3'-terminus region of coagulase gene was performed using specific oligonucleotide primers designed by the ZAO Eurogene Company (Russia) described in the literature [10]. Ready-to-use ScreenMix-HS mastermixes (ZAO Eurogene, Russia) were used for PCR. Genomic DNA was amplified in 250 µl of reaction mixture comprising 5 µl of ScreenMix-HS amplification mix, 5 µl of primers, 5 µl of deionized water and 10 µl of DNA. Amplification was performed in automatically programmable Tercycle thermocycler (OOO NPO DNA-technology, Russia) according to the following DNA amplification scheme: predenaturation – at 94 °C for 2 min (1 cycle); denaturation, annealing, extension– at 94 °C for 30 sec., 57 °C for 30 sec., 72 °C for 20 sec. (30 cycles); final extension – at 72 °C for 5 min (1 cycle).

PCR-RFLP. Coa-PCR-RFLP analysis was carried out with method proposed by J. V. Hookey et al. [10] using *Cfo1* restriction endonuclease (Promega, США) according to attached instruction. Further analysis was carried out by determination of resulting amplicon sizes, restriction fragment number and length. *Coa*-PCR-RFLP-patterns were presented in the form of numerical code where the first number (before slash) shows PCR product size and the second number (after slash) shows *Cfo1* restriction fragment sizes.

Gel-electrophoresis. Amplification products were visualized with 2% agarose gel electrophoresis (agarose, Sigma, USA) in Tris-acetate-EDTA buffer (TAE) at voltage gradient of 150 V for 20 min (electrophoresis of restriction products at 80 V for 1 hour). The gel was stained with ethidium bromide solution (0.5 µg/ml) and was examined in UV transilluminator upon the electrophoresis completion (wave length – 254 nm). Amplicon sizes were determined using 100–1200-base pair DNA marker (ZAO Eurogene, Russia). Photos of the reaction results in UV light were made.

RESULTS AND DISCUSSION

All tested Staphylococcus cultures produced lecithinase, demonstrated hemolytical activity, had plazma-coagulating properties (rabbit blood clotting ability) and fermented mannitol under anaerobic conditions. In smears prepared from cultures grown in nutrient medium Staphylococci appeared as characteristic accumulations of Gram-positive cocci (often grape-like clusters). Isolates definitely identified as *S. aureus* based on results of biochemical identification tests with Multimicrotests for

biochemical identification of staphylococci (MMT C) and latex agglutination tests were selected for molecular genetic examination.

Tests for detection of *mecA* gene, bacterium genetic determinant responsible for Staphylococcus resistance to β-lactam antibiotics of penicillin group, showed that all *S. aureus* cultures lacked the said gene, i.e. the cultures were methicillin-susceptible (MSSA).

All *S. aureus* strains are known to produce coagulase. 115 *S. aureus* isolates were examined for coagulase gene polymorphism and analyzed for restriction fragment polymorphism length. As a result, 4 amplicons of different length, 600, 700, 800 and 900 bp, were generated that was indicative of structural differences in coagulase gene of tested isolates (Table 1).

S. aureus isolates (46%) having 900 base-pair variable site in coagulase gene were predominate in the tested samples. 700 base-pair amplicon was the second most frequently detected, it was detected in 41 isolates (36%). In 16 and 5 isolates (14 and 4%) coagulase gene size was 600 and 800 bp, respectively. Table 1 shows the frequency of *coa*-amplicon detection in *S. aureus* recovered from various organs.

According to the data given in the Table, 900 base-pair and 700 base-pair *coa*-amplicons were more often detected in *S. aureus* (33 and 30 isolates, respectively) recovered from lungs of monkeys died of pneumonia. 600 base-pair amplicon was detected in 14 *S. aureus* isolates recovered from lungs as well as from liver and parenchymal fluid.

RFLP typing of the above-said isolates was the next step. Coagulase gene PCR products were subjected to restriction fragment length polymorphism analysis and digested with *Cfo1* endonuclease into separate fragments. Number restriction patterns varied from one to four, a restriction fragment size varied from 80 bp to 600 bp (Table 2).

PCR-RFLP analysis of amplicons revealed 23 types of coagulase gene hence 23 different *S. aureus* strains were found to circulate in monkeys kept on the monkey farm. Isolates of each group had amplicons of the same size and specific restriction sites. Lack of differences in the amplicon sizes indicated lack of differences in number of nucleotide repeats and similar restriction sites indicates the structural similarity of such repeats.

Two types of *Cfo1* restrictions were detected among isolates generating 600 base-pair amplicons. 700 base-pair amplicons showed 8 types of restrictions. Twelve isolates out of the selected ones were not digested with *Cfo1*, i.e. they lacked restriction sites for this endonuclease (700/700 bp pattern). 800 base-pair amplicons showed 3 types of restrictions. RFLP analysis of 900 base-pair amplicon revealed 10 types of restrictions.

Analysis of RFLP patterns revealed that 900/80-250-300 bp pattern (XIV type of PCR-RFLP) detected in 28 *coa*-amplicons was the most common. The second largest group (n = 14) comprised *S. aureus* isolates generating 700 base-pair amplicons and showing 4 restriction fragments 80-150-180-300 bp in length (III type of PCR-RFLP). Nine (9) *coa*-amplicons were found to have 600/150-250 bp pattern (I type of *coa*-gene). The following *coa*-patterns: 600/80-150-300 and 900/100-250-350 bp (II and XV *coa*-types, respectively) were detected in 7 amplicons. Thirteen (13) RFLP-patterns of coagulase gene were the rarest since they were detected in one or two isolates.

During the study, an attempt to establish association between coagulase gene structure (*coa*-PCR-RFLP-patterns) and ability of the said isolates to cause pneumonia in monkeys was made. Seven types of *S. aureus* coagulase gene were detected in lungs only. The said isolates were the most likely show tropism to lung tissue. Isolates with XIV, III and I types of *coa*-gene were predominant among the isolates detected in pneumonia cases. *S. aureus* with eleven types of coagulase gene can be classified to invasive isolates since they were successfully detected in tissues of various tested organs. Hence, Staphylococcus infection is mediated by genotypically heterogeneous population of the agent.

Table 1
Frequency of *coa*-amplicon detection

Source of isolation	*coa*-amplicon length (base pairs)	Number of isolates
Lung (n = 79)	600 700 800 900	14 30 2 33
Liver (n = 10)	600 700 900	1 5 4
Spleen (n = 6)	800 900	1 5
Kidney (n = 5)	700 900	1 4
Lymph node (n = 8)	700 800 900	3 2 3
Uterus (n = 1)	700	1
Parenchymal fluid (n = 2)	600 900	1 1
Puss (n = 2)	900	2
Eye (n = 1)	700	1
Pharynx (n = 1)	900	1
Total		**115**

Analysis of data on coagulase gene structure polymorphism showed that some *coa*-patterns of tested *S. aureus* occur in the certain monkey species. For example, 700/100-200-bp pattern (VIII type of *coa*-gene) was detected in *S. aureus* isolated from lungs of two hamadryas baboons; 800/150-200-300 bp pattern (XIII type of *coa*-gene) – in *S. aureus* isolated from a lymph node of a monkey of the same species; 700/250-600 bp pattern (VI type of *coa*-gene) – in *S. aureus* isolated from lungs of three crab-eating macaques; 700/150-550 bp pattern (X type of *coa*-gene) – in *S. aureus* isolated from liver of a crab-eating monkey. Thus, it can be suggested that there are some *S. aureus* strains circulating in the certain monkey species but this requires further investigation.

CONCLUSION

S. aureus-associated infection is relatively common in monkeys living on Adler monkey farm. High diagnostic titres yielded by plated microbes from body organs,

Table 2
RFLP-PCR of coagulase gene of MSSA isolates recovered from monkeys

Source of isolation	Number of isolates	*coa*-PCR-RFLP pattern	PCR-RFLP-type
Lung ($n = 9$)	9	600/150-250	I
Lung ($n = 3$), liver ($n = 4$)	7	600/80-150-300	II
Lung ($n = 13$), spleen ($n = 1$)	14	700/80-150-180-300	III
Lung ($n = 5$), liver ($n = 3$), lymph node ($n = 2$), kidney ($n = 1$), eye ($n = 1$)	12	700/700	IV
Lung ($n = 4$), lymph node ($n = 1$)	5	700/150-450	V
Lung	3	700/250-600	VI
Lung	2	700/80-150-400	VII
Lung	2	700/100-200-350	VIII
Lung, uterus	2	700/200-500	IX
Liver	1	700/150-550	X
Lung, spleen	2	800/80-180-300	XI
Lung, spleen	2	800/150-500	XII
Lymph node	1	800/150-200-300	XIII
Kidney ($n = 16$), lymph node ($n = 2$), spleen ($n = 5$), kidney ($n = 2$), liver ($n = 1$), pus ($n = 1$), pharynx ($n = 1$)	28	900/80-250-300	XIV
Kidney ($n = 1$), lung ($n = 4$), Lymph node ($n = 1$), pus ($n = 1$)	7	900/100-250-350	XV
Lung ($n = 4$), parenchymal fluid ($n = 1$)	5	900/80-140-300-500	XVI
Lung ($n = 2$), liver ($n = 1$)	3	900/100-250-500	XVII
Lung	2	900/80-150-250-450	XVIII
Lung	2	900/100-180-350	XIX
Lung	2	900/140-300-500	XX
Liver	1	900/80-220-480	XXI
Kidney	1	900/100-180-220-500	XXII
Liver	1	900/250-500	XXIII

tissues, fluids and cavities that normally should be sterile are considered to be a confirmation of the said microorganism etiological role in the disease development. The study showed that *S. aureus* recovered from monkeys had pathogenic properties and belonged to MSSA, since they lacked *mecA* gene based on results of PCR amplification using fluorescent-labelled specific primers.

Coagulase production is an important phenotypic characteristic used for *S. aureus* identification. It is known that RFLP analysis is a gold standard for molecular typing of *S. aureus* isolates as it is considered to be technically simple, easily reproducible method that has high specificity and therefore is successfully used for *S. aureus*—caused infection epidemiology investigation [5]. Coagulase gene 3'-terminal region variability is used as a basis for typing of the isolates recovered from humans and animals [8, 12, 13, 15]. According to A. Salehzadeh et al., the difference in coagulase gene types can be accounted for by geographical variability [12]. The said polymorphism is also accounted for by different mutations due to which a part of 3'-terminal region is deleted or several new nucleotides are inserted and as a consequence the size of coagulase gene changes.

Coagulase gene polymorphism-based genetic typing of *S. aureus* isolated from monkeys confirmed high heterogeneity of *S. aureus coa*-gene. Examination of coagulase gene fragment with gel-electrophoresis revealed amplicons of four sizes – 600, 700, 800, 900 bp in length that was indicative of structural differences in the said gene in tested isolates. However, 900 base-pair *coa*-gene were predominate in the selected isolates (46%). PCR-RFLP analysis revealed 23 genovariants of MSSA. Despite of detection of multiple *coa*-gene genotypes only some of them were predominant. Most of *S. aureus* strains isolated from monkeys

belonged to XIV, III and IV types. *S. aureus* showing the following three PCR-RFLP patterns of *coa*-gene were the most often detected in pneumonia cases: XIV, III and I. Hence, the said isolates showed tropism to lung tissues.

Obtained data were consistent to results of analyses carried out by other authors confirming existence of several genotypes of *S. aureus* isolated from humans and animals [8, 11, 15]. Coagulase gene polymorphism can be used as a tool for assessment of Staphylococcus infection situation in monkey population kept on the monkey farm. Further investigation of predominant *S. aureus* genovariants, identification of their common characteristics (for example antimicrobial resistance) can be also used for development of measures for *Staphylococcus*-associated pneumonia prevention and treatment in monkeys.

Conflict of interest. Authors declare no conflict of interest.

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