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Coagulase gene and *agr* complex polymorphism-based genotyping of *Staphylococcus aureus* isolated from lower primates

V. A. Kalashnikova
FSBSI "Research Institute of Medical Primatology" (FSBSI "RIMP"), Sochi, Russia;
https://orcid.org/0000-0002-1574-8674, e-mail: vikky.aw@gmail.com

SUMMARY

Staphylococcus aureus is a pathogenic microorganism causing a great number of diseases in humans and animals. Many researches on genotyping *Staphylococcus aureus* isolated from humans and mastitis affected cowsare performed, but no foreign reports on typing of *Staphylococcus aureus* detected in monkeys have been found. Staphylococcus-induced infections are however widely spread in primates. The paper demonstrates results of molecular and genetic examination of *Staphylococcus aureus* isolated from lower primates. The examination was based on typing of coagulase gene and polymorphic locus of *arg* gene that regulates expression of pathogenicity-associated genes. Structures of coagulase gene (*coa*) and polymorphic types of regulatory gene (*agr*) were studied in 145 *Staphylococcus aureus* isolates recovered from various monkey species. The studies resulted in singular coagulase gene fragments of four dimensions: 600, 750, 800 and 900 bps. Following *AluI* endonuclease restriction results *Staphylococcus aureus* was classified in seven different *coa*-types. Coagulase gene of genotype VII predominated (31.7%), genotype II was detected less frequently (9.7%). Each *Staphylococcus aureus* isolate is specified by a definite coagulase gene restriction profile; therefore, at least seven *Staphylococcus aureus* strains are currently circulating in the monkeys in the monkey facilities. Herewith, those staphylococci that bear genotype VII coagulase gene are invasive as they are isolated from various organs and pus as well as from feces and nasal cavities of the animals. Analysis of the study results demonstrated that bacteria of this species could be transmitted between different monkey species. Apart from human *Staphylococcus aureus*, in whose genome *agrI* prevails, *agrIV* prevailed in the isolates outlined in this paper (59.3%); *agrII* and *agrIII* were detected in 5.5 and 2.1% of the isolates, respectively.

Key words: monkeys, *Staphylococcus aureus*, coagulase gene, regulatory gene, *agr* groups

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For correspondence: Victoria A. Kalashnikova, Candidate of Science (Biology), Leading Researcher, FSBSI "RIMP", 354376, Russia, Krasnodar Krai, Sochi, s. Veseloye, ul. Mira, 177, *e-mail: vikky.aw@gmail.com*.

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Генотипирование *Staphylococcus aureus*, выделенного у низших приматов, на основе полиморфизма коагулазного гена и генов комплекса *agr*

В. А. Калашникова
ФГБНУ «Научно-исследовательский институт медицинской приматологии» (ФГБНУ «НИИ МП»), г. Сочи, Россия;
https://orcid.org/0000-0002-1574-8674, e-mail: vikky.aw@gmail.com

РЕЗЮМЕ

Золотистый стафилококк (*Staphylococcus aureus*) – патогенный микроорганизм, вызывающий большое количество заболеваний у человека и животных. Проведено много исследований по генотипированию *Staphylococcus aureus*, выделенных у людей и при маститах у коров, но данных по типированию *Staphylococcus aureus*, обнаруженного у обезьян, в зарубежной литературе не найдено. Однако инфекции стафилококковой природы у приматов распространены широко. В статье представлены результаты молекулярно-генетического изучения золотистых стафилококков, изолированных от низших обезьян, на основе типирования коагулазного гена и полиморфного локуса *agr*, являющегося регулятором экспрессии генов патогенности. Проведено

исследование структуры коагулазного гена (*coa*) и полиморфных типов регуляторного гена (*agr*) у 145 изолятов *Staphylococcus aureus*, выделенных от обезьян разных видов. Получены одиночные фрагменты коагулазного гена четырех размеров: 600, 750, 800 и 900 п. н. По результатам рестрикции эндонуклеазой *AluI* изученные золотистые стафилококки классифицированы на семь различных *coa*-типов. Наиболее часто обнаруживали VII генотип (31,7%), реже – II генотип коагулазного гена (9,7%). Для каждого изолята *Staphylococcus aureus* характерен определенный профиль рестрикции коагулазного гена, следовательно, среди обезьян питомника циркулирует как минимум семь штаммов золотистого стафилококка. При этом стафилококки, содержащие коагулазный ген VII генотипа, являются инвазивными, так как выделены из различных органов и гноя, а также фекалий и носовой полости животных. Анализ результатов исследования показал, что между разными видами обезьян возможна передача бактерий данного вида. Установлено, что, в отличие от обнаруженных у людей изолятов *Staphylococcus aureus*, в геноме которых преобладает *agrI*, у изученных в настоящей работе изолятов превалирует *agrIV* (59,3%). Группы *agrII* и *agrIII* детектированы у 5,5 и 2,1% изолятов соответственно.

Ключевые слова: обезьяны, *Staphylococcus aureus*, коагулазный ген, регуляторный ген, группы комплекса *agr*

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Для корреспонденции: Калашникова Виктория Алексеевна, кандидат биологических наук, ведущий научный сотрудник ФГБНУ «НИИ МП», 354376, Россия, Краснодарский край, г. Сочи, с. Веселое, ул. Мира, 177, *e-mail: vikky.aw@gmail.com.*

INTRODUCTION

Staphylococcus aureus is the cause of human and animal diseases including benign infections of the skin and soft tissues [1, 2]. Danger of the pathogen involves its capacity to cause severe infections to the extent of lethal ones (pneumonia, sepsis, etc.). This is due to the action of a large number of virulence factors produced by it, such as staphylokinase, adhesins, hemolysines, leukocidins, enterotoxins, etc. [2, 3], whose expression is regulated by aspecific system – additional regulator gene *agr* [2, 4–7]. Although this locus is a conservative one, it consists of polymorphic hypervariable fragment used for classifying *St. aureus* strains to one of four groups (polymorphic types) – *agrI*, *agrII*, *agrIII*, *agrIV* – by polymerase chain reaction (PCR) [4, 8]. Some researchers insist that *agr*-groups influence the virulence of the strains and disease progression [9].

In the late twentieth century, molecular typing methods became applicable for the research of different pathogenic microorganisms, and they allowed for the detection of epidemically significant strains. Such methods allowed detection of genetic determinants of *St. aureus* virulence and therefore it was established that this microorganism belongs to heterogenic and polymorphic species, in which pathogenicity genes are located in the chromosomal pathogenicity islands, chromosomal cassettes and prophages. In different strains, these genetic elements are present as allelic forms and specified by different degree of mobility [4]. Horizontal transfer of genes located on the mobile genetic elements results in the genetic diversity within *St. aureus*.

One of the key *St. aureus* virulence factors is coagulase – extracellular product of the strains and it stimulates prothrombin thus resulting in blood clotting. Coagulase gene *coa* is genetically variable, i.e. polymorphic [10, 11]. Coagulase typing was developed as one of the molecular tools used for identification and differentiation of *St. aureus* strains [12, 13]. Coagulase gene amplification is considered simple and accurate typing tool for *St. aureus* isolated from different sources and bearing relevant information on the genetic background of the isolates [14, 15]. This method is based on the detection of variability and polymorphism resulted from the mutations occurring on coagulase gene 3´-terminal bearing tandem arrays of 81 base pairs (bp) and changing the size of the gene [16–19]. DNA fragments associated with the coagulase gene variable region are subjected to PCR-amplification followed by restriction enzyme (endonuclease) cleavage and analysis of the different restriction fragment lengths or patterns (PCR-RFLP).The differentiation of isolates is based on the data on the number and size of such fragments. Therefore, examination of the polymorphism of such *St. aureus* virulence genes as coagulase and regulatory ones can be of diagnostic importance [12, 20]. Coagulase gene genotypes are well studied in case of *St. aureus* spp. isolated from humans [21]. Many studies were also devoted to the examination of the *coa*-gene polymorphism in *St. aureus* spp. Isolated from mastitis-affected caws and milk [1, 6, 11, 15, 22–25].These studies demonstrated that different *St. aureus* genotypes can be isolated from mastitis-affected cows not only in different geographical locations but within the same herd [6]. According to the published data, the strains can be transmitted between humans and different animals including monkeys [24]. Human strains of *St. aureus* colonize and infect monkeys, both captured and wild [26, 27]. Acquisition and loss of genes located on mobile genetic elements are considered the main factor of the microbe adaptation after transmission between the hosts [26].

No research data on coagulase gene polymorphism and allelic variants of the regulatory gene of *St. aureus* isolated from various monkey species were found in published reports.

The purpose of this work is to carry out genotyping of *St. aureus* isolates recovered from various monkey species basing on the coagulase gene polymorphism and polymorphic types of the regulatory gene.

MATERIALS AND METHODS

We studied 145 *St. aureus* isolates recovered as are result of bacteriological tests of 33 live and 100 dead monkeys of various species (*Macaca mulatta* – 51, *Macaca fascicularis* – 33, *Papio hamadryas* – 33, *Papio Anubis* – 9, *Chlorocebus sabaeus* – 3, *Macaca nemestrina* – 3, *Cebus capucinus* – 1), kept in captivity in the Adler monkey colony. Monkeys of both sexes (♀ – 69, ♂ – 64) and of different age (from day 0 to 35 years of age) were tested. Swabs from the nasal mucosa ($n = 11$), pharynx ($n = 1$), urethra ($n = 1$), wound pus ($n = 2$) were taken from living monkeys for the study. Fecal samples were collected from rectum ($n = 18$). In case of dead animals, test materials included pieces of organs and biological fluids collected during post-mortem examination (lungs – 66, liver – 11, spleen – 12, kidney – 6, lymph node – 9, uterus – 1, caecum 4, pus – 2, biological fluid – 1).

All animal experiments were performed in strict compliance with interstate standard on laboratory animal keeping and handling GOST 33215-2014, adopted by Interstate Council on Standardization, Metrology and Certification and pursuant to the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

The material was inoculated to the salt agar with egg yolk and 5% blood agar and incubated at 37 °C for 24–48 hours. The grown staphylococcus colonies were examined morphologically (based on description of colonies, their pigmentation, Gram staining) and biochemically, taking note of lecithinase activity in salt agar with egg yolk, hemolytic activity in blood agar as well as their blood clotting activity. The species were identified using commercial test-kits 'Multimicrotests for biochemical identification of staphylococci (MMTS)' (OOO NPO Immunotex, Russia). Until further tests, the *St. aureus* isolates were stored at minus 20 °C.

DNA extraction and *agr*-typing were performed as described above [28].The coagulase gene was detected according to the procedure described in the previous paper [29]. Coagulase gene structural polymorphism was examined according to J. V. Hookey et al. [30] using *AluI* restriction endonuclease (New England BioLabs, USA) according to the following procedure: 5 µl 10× NEBuffer, 34 µl of deionized water, 10 µl of DNA, 1 µl of *AluI*. The mixture was incubated for 1 hour at 37 °C. Further analysis was based on the obtained amplicons, amount and length of the restriction fragments. *Coa*-PCR-RFLP-patterns were demonstrated as numerical code: first number (before slash) corresponds to the PCR-product length; next (after slash) lengths of restriction *AluI*-fragments are designated.

The amplification products were visualized in Tris-acetate buffer (TAE) using 2% agarose gel (Sigma, USA) stained with ethidium bromide solution (0.5 µg/ml) at 130V gradient for 50 min (electrophoresis of the restriction products occurred at 80V for 1 hour 35 min). Upon the electrophoresis completion, the results were visualized in UV transilluminator (wave length 254 nm). The size of the amplicons was determined using 100-1200 bp DNA ladder (Evrogen, Russia). There action results were UV photographed.

Table 1
Specification of coagulase gene PCR-products (amplicons) of *St. aureus* isolated from monkeys

coa-type	PCR-product size (~ number of base pairs)	Number of isolates (%)	Number of PCR-patterns
1	600	42 (29%)	1
2	750	17 (12%)	1
3	800	19 (13%)	3
4	900	67 (46%)	3

RESULTS AND DISCUSSION

Four types of amplicons were identified following the results of the coagulase gene amplification (Table 1). The table demonstrates that the majority of the isolates (46%) contained 900 bp type IV coagulase gene.

Use of *AluI* endonucleases allowed for identification of seven different coagulase gene restriction profiles (genotypes) in the tested isolates that provided from one to three restriction fragments varying size – from 80 to 750 bp (Table 2).

Table 2
Frequency of monkey's *St. aureus* coagulase gene and *agr* detection by genotypes and types

coa-gene genotype (restriction profile)	*coa*-PCR-RFLP-patterns, bp	*agr*-type (number)	Total
I	600/600	*agrI* – 6 *agrIV* – 22	28 (19.3%)
II	600/300	*agrI* – 11 *agrII* – 2 *agrIV* – 1	14 (9.7%)
III	750/750	*agrI* – 5 *agrII* – 1 *agrIV* – 11	17 (11.7%)
IV	800/400-220-80	*agrI* – 4 *agrIV* – 15	19 (13.1%)
V	900/220-180-80	*agrI* – 8 *agrII* – 2 *agrIII* – 1 *agrIV* – 7	18 (12.4%)
VI	900/450-220-80	*agrI* – 3	3 (2.1%)
VII	900/550-220-80	*agrI* – 11 *agrII* – 3 *agrIII* – 2 *agrIV* – 30	46 (31.7%)

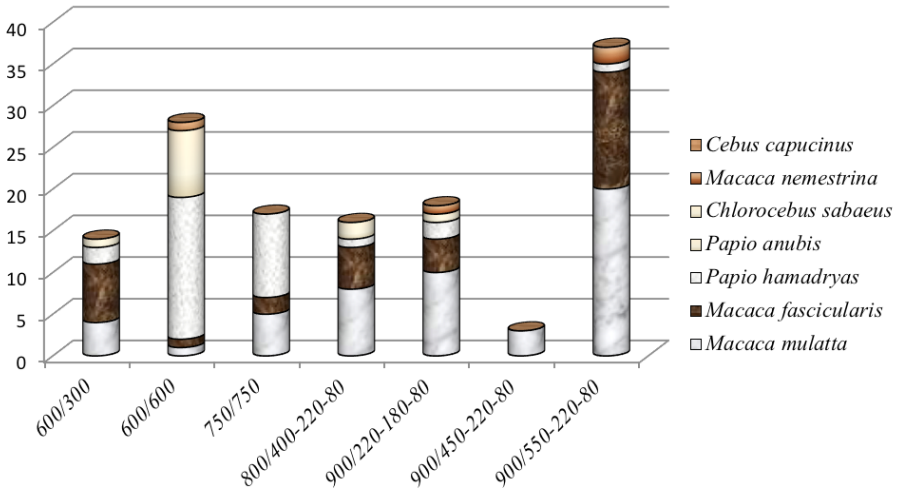

Fig. Coagulase gene restriction profiles of St. aureus isolated from different monkey species

Wherein, similar length of amplicons and specific restrictions were typical for each group. Absence of any difference in the amplicons size is indicative of the absence of the difference in the number of nucleotide repeats, while similar restriction sites indicate structural similarity of these repeats. The majority of *St. aureus* isolates belonged to type VII (31.7%); less frequently genotype VI coagulase gene was detected (2.1%).

It should be noted that the restriction profile of 600/600 bp (*coa*-gene of genotype I) was frequently observed in *St. aureus* isolated from baboons: in 25 out of 42 tested isolates (Figure). Detection of coagulase gene with such structure in two macaques and capuchin can be explained by the fact that these animals were quarantined together with baboons (*St. aureus* infected or carriers) that resulted in their infection with this isolate. It can be therefore supposed that *St. aureus* circulates between the monkeys at the quarantine facilities.

Pattern 900/550-220-80 (genotype VII) was more frequently detected in *St. aureus* isolated from macaques: in 36 out 87 tested isolates. Only three rhesus macaques demonstrated coagulase gene having 900/450-220-80 pattern (*coa*-gene of genotype VI). While each isolate is specified by its own restriction profile, the obtained data demonstrate that at least seven *St. aureus* isolates circulate in monkeys kept in Adler monkey colony.

St. aureus strain containing genotype VII *coa*-gene (900/550-220-80) is invasive because it is isolated from all organs and pus (Table 3).

On the other hand, the majority of *St. aureus* isolates detected in feces and nasal cavities of clinically healthy monkeys possess genotype I of coagulase gene (pattern 600/600). *St. aureus* with coagulase gene of genotype III and VII were most frequently isolated from the lungs of dead monkeys with diagnosed pneumonia. Some researchers supposed that *St. aureus* with prevailing coagulase genotype are more resistant to neutrophils as compared to the ones with rare genotypes [23].

Agr-typing of *St. aureus* demonstrated that *agr* of group IV was detected in more than half of the isolates, while *agr* of groups II and III were detected rarely (Table 4).

The table demonstrates that *agrIII* was detected in two *St. aureus* isolates recovered from the liver and in one isolate recovered from the spleen. In none of the 66 *St. aureus*

Table 3
Types of coagulase gene of *St. aureus* isolated from biomaterials of monkeys

Type of *coa*-gene	Test material (amount)								
	lung ($n = 66$)	liver ($n = 11$)	kidney ($n = 6$)	spleen ($n = 12$)	lymph node ($n = 9$)	caecum ($n = 4$)	pus ($n = 4$)	feces ($n = 18$)	nasal mucosa ($n = 11$)
I	8	1	1	0	0	1	1	9	5
II	11	1	0	0	0	0	1	1	0
III	12	1	0	1	0	2	0	0	1
IV	8	1	1	3	2	0	0	3	0
V	8	1	0	1	2	0	0	3	3
VI	0	1	1	1	0	0	0	0	0
VII	19	5	3	6	5	1	2	2	2

Table 4
Regulatory gene groups identified in *St. aureus* isolated from different biomaterials of monkeys

Test material	*agr*-type, quantity (%)				Total
	I	II	III	IV	
Lung	22 (33.3%)	3 (4.6%)	0	41 (62.1%)	66
Liver	4 (36.4%)	0	2 (18.2%)	5 (45.4%)	11
Kidney	2 (33.3%)	0	0	4 (66.7%)	6
Spleen	4 (33.3%)	0	1 (8.3%)	7 (58.4%)	12
Lymph node	4 (44.5%)	0	0	5 (55.6%)	9
Uterus	0	0	0	1	1
Pus	1	2	0	1	4
Body fluid	0	0	0	1	1
Caecum	2	1	0	1	4
Feces	5 (27.7%)	1 (5.6%)	0	12 (66.7%)	18
Nasal mucosa	4	1	0	6	11
Throat	0	0	0	1	1
Urethra	0	0	0	1	1
Total	48 (33.1%)	8 (5.5%)	3 (2.1%)	86 (59.3%)	145

isolated from the lungs of the monkeys the regulatory gene of group III was detected, and the isolates recovered from the liver and spleen did not demonstrate *agrII*.

The frequency of detection of *agr* complex genes in *St. aureus* isolates with different types of coagulase gene is shown in Table 2. Analysis of *agr*-typing results demonstrated that in all isolates with Type VII *coa*-gene only group I regulatory gene was detected. Group IV *agr* prevailed in all remaining staphylococci.

CONCLUSION

Use of coagulase gene typing, therefore, allowed for the detection of different genotypes among *St. aureus* isolated from various monkey species. This fact confirms that *St. aureus* has certain heterogenicity in 3´-terminal region of *coa*-gene. Four types of 600–900 bp coagulase gene and seven its restriction profiles with the fragments varying from 80 to 550 bp in length were detected during the studies. Type IV coagulase gene of 900 bp in length was the most wide spread and it was detected in 46% of the isolates, as well as genotype VII *coa*-gene restriction (pattern 900/550-220-80 bp) detected in 31.7% of *St. aureus*. The obtained results demonstrate that pneumonia in monkeys are induced by *St. aureus* strains exhibiting wide coagulase gene polymorphism; however, the majority of *St. aureus* isolates recovered from the lungs (19 of 66) were classified in coagulase gene restriction profile VII (28.8%). There search results suggest that at least seven *St. aureus* isolates circulate in monkeys kept in the monkey colony and these microbes are able to induce pneumonia; they can be part of the concomitant microflora during intestinal diseases (thus persisting both in the intestines and internal organs) and colonize nasal cavity of the clinically healthy animals.

The *agr*-typing demonstrated that group IV regulatory gene prevailed in *St. aureus* isolated from monkeys, while *agrI* prevailed in *St. aureus* isolated from humans, and *agrI* and *agrIII* prevailed in *St. aureus* recovered from the mastitis-diseased cows [4, 6, 25]. Spread of other *St. aureus* virulence determinants is to be further studied, and the above mentioned genes are to be sequenced that will allow to identify whether they belong to the clonal lineages of *St. aureus* circulating in monkeys, to compare them with the data resulted from the examination of the strains isolated from humans and other animals and to assess the epidemic situation in the monkey facilities in terms of this bacterial pathogen.

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INFORMATION ABOUT THE AUTHOR / ИНФОРМАЦИЯ ОБ АВТОРЕ

Victoria A. Kalashnikova, Candidate of Science (Biology), Leading Researcher, FSBSI "RIMP", Sochi, Russia.

Калашникова Виктория Алексеевна, кандидат биологических наук, ведущий научный сотрудник ФГБНУ «НИИ МП», г. Сочи, Россия.