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CHARACTERISTICS OF COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM CENTRAL VENOUS CATHETERS OF NEONATES IN AN INTENSIVE CARE UNIT: FORMATION OF BIOFILMS AND GENETIC POPULATION STRUCTURE IDENTIFIED BY MULTILOCUS SEQUENCE TYPING

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Aim *To investigate the ability of hospital-associated populations of S. epidermidis to form biofilms on the inner surface of central venous catheters (CVC).*
Material and methods *The study analyzed 150 samples of CVCs obtained after catheterization of neonates.*
Results *Fifty three catheters were colonized by conditional pathogens, and 50 samples were contaminated by a single strain. Isolates of S. epidermidis were present in 68% of samples. They were assigned to 12 different sequence types, most frequently to ST59 (19 isolates, 56%). The icaADBC operon genes, which are involved in the production of the polysaccharide intercellular adhesin (PIA), were not detected in the majority of isolates.*
Conclusion *Most of CVCs were colonized by S. epidermidis (62% of isolates) with sequence types belonging to the CC2 clonal complex, thus indicating that they are hospital acquired. A group of isolates with an increased ability to adhere to plastics was identified. Some isolates were found to be ica-operon negative, but capable of forming a biofilm on the catheter surface by an ica-independent mechanism.*

Keywords: *coagulase-negative staphylococci, biofilms, catheter-associated infections, newborn children.*

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Authors declare lack of the possible conflicts of interests.

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The progress of modern medicine, primarily in surgery and critical care, has been accompanied by increasing implementation of invasive interventions using implanted devices (catheters, prostheses) manufactured from polymeric materials. This development has resulted in a significantly increased risk of device-associated hospital acquired infections.

Catheter-associated bloodstream infections (CAIs) are one of the most common complications of central venous catheterization in long-term hospital patients. CAIs are accompanied by bacteremia, dissemination of the pathogens, and are always associated medical care; therefore hospital acquired conditional pathogens are often the etiological agent [1]. The mortality attributable to CAIs estimated by various authors is as high as12-25% [2].

One of the main causative agents of CAIs are coagulase-negative staphylococci (30-50% of cases) and in particular *Staphylococcus epidermidis* [3, 4]. These bacterial species cause at least 22% of bloodstream infections in intensive care units [3].

An important feature of *S. epidermidis* is its ability to form biofilms and colonize biomaterials, including catheters. A bacterial biofilm is defined as surface-attached microbial communities encased in an extracellular matrix. Bacteria in biofilms have a higher tolerance against the immune response and antimicrobial agents, which significantly reduces the effectiveness of anti-bacterial therapy. Thus, the resistance of biofilm-forming microorganisms may be 100-1000 times higher than that of the same bacteria in the planktonic state [5]. This resistance has been attributed to some factors such as the persistence of biofilm-forming strains with a highly retarded metabolism, filtering ability of the biopolymer matrix that prevents antibiotics to diffuse through biofilms. At the same time, the formation of biofilms in the foci of infection in the human body leads to a chronic process that may result in treatment failure [6].

An extracellular matrix of biofilms is composed of exopolysaccharides, proteins and extracellular DNA [7]. The main component of biofilms formed by *S. epidermidis* is the β-1,6-N-acetylglucosamine polysaccharide polymer, also called polysaccharide intercellular adhesin [8, 9]. Also, the formation of biofilms is mediated by proteins of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family [10] and autolysins [8].

The problem of biofilm growth on the surface of catheters is especially important in intensive care for neonates with low and extremely low birthweight [11]. Physiological immunodeficiency in newborns and the immaturity of preterm infants' organ systems and receptor apparatus (Toll-like receptors) predisposes them to severe infections caused by conditional pathogens with low pathogenic potential, such as *S. epidermidis* that colonize CVCs. According to MLST data from a large multicenter study reported by M. Miragaia et al. in 2007, hospital population of *S. epidermidis* was represented by related strains belonging to one major clonal complex, for which ST2 was a direct ancestor [12]. This gives rise to the assumption that the representatives of this clonal complex have an evolutionary advantage over other strains of these species, which allows them to survive and occupy a dominant position in the hospital environment. Given that coagulase-negative staphylococci, in contrast to *S. aureus*, have a very small set of pathogenicity factors [3], their ability to survive in a hospital environment can be attributed to a good adhesion on the surfaces of medical devices that are indispensable in a modern hospital. Considering this, this study aimed to investigate the ability of hospital-associated populations of *S. epidermidis* to form biofilms on the inner surface of CVCs.

Material and methods

A total of 150 CVCs was collected at the V. I. Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology. Immediately after removal of the catheter near the neonatal incubator in the neonatal intensive care unit (ICU) of the Center, its distal end was divided into two fragments with scissors in line with requirements for the aseptic processing of medical devices. Pieces of CVCs were placed in tubes with dextrose broth (HiMedia, India) and 10% formalin. The tube with the dextrose broth was incubated for 24 hours at 37°C, the formalin tubes were sent for microscopic examination and were stored at 6-8°C. In the case of turbidity of the dextrose broth, it was placed onto 5% sheep blood agar media (Oxoid, UK) on Petri dishes. Colonies grown on agar were identified by direct mass spectrometry profiling of bacterial lysate [13]. The mass spectra were obtained using an Autoflex III time-of-flight mass spectrometer (Bruker Daltonics, Germany) equipped with a 337 nm nitrogen laser, and were evaluated with the MALDI Biotyper 3.0 software (Bruker Daltonics, Germany).

The surface of the catheters was visualized with scanning electron microscopy (SEM): 1. control - an intact 24G catheter (Vygon GmbH & Co.KG, Germany); 2. a catheter taken from a newborn 6 days after catheterization, not colonized by microorganisms according to a microbiological study; 3. a catheter colonized *by S. epidermidis* and taken from a newborn 4 days after catheterization.

S. epidermidis isolates were analyzed using MLST to detect genes responsible for the formation of biofilms and evaluate their biofilm forming ability *in vitro* and *in vivo*.

To isolate the bacterial culture DNA, a set "DNA Express" (TU-9398-450-17253567-03) (Lytech Co. Ltd., Russia) was used according to the manufacturer's instructions. DNA samples were stored at -20 ° C.

The typing of *S. epidermidis* isolates was performed by multilocus sequence typing using the standard MLST scheme (http://sepidermidis.mlst.net). Detection of the of icaABCD operon genes responsible for the synthesis of the polysaccharide intercellular adhesin for the Staphylococcus genus was carried out by amplification of the corresponding genes by polymerase chain reaction using three different techniques [14-16].

The presence of genes encoding the MSCRAMM family (Ebp, SdrH) and AtlE, Aap, and Ebh proteins was confirmed by amplification using the primers shown in the table. The primers were selected using Oligo 6.71 software (Molecular Biology Insights, Inc., USA).

The ability of isolates to adhere to plastic and form biofilms was evaluated by staining the biofilm with crystal violet (in vitro) and by SEM (in vivo). Staining of the biofilms with crystalline violet was performed as follows: the isolates were grown in a liquid nutrient medium (tryptone soya broth, Oxoid) overnight at 37°C. The 24-h cultures of the studied strains were added with tryptone soya broth to prepare suspensions with an optical density of 0.5 McFarland. Aliquots of bacterial suspensions (10 µl) were added to wells of a 96-well plate (Greiner bio one, Austria) filled with tryptone soya broth (200 µl) and incubated at 37°C for 24 hours. After that, the culture liquid was carefully removed, the wells

Table. List of primers used to detect *S. epidermidis* genes involved in biofilm formation

genes	Sequence 5'-3'	Amplicon length, n	Temperature, °C
aap	CAACGAAGACAGAAGAAGG TGAACATAAAGCCCCAACC	654	56
atl	CTACTCAATCAACACCAGC AAATCCTTAGCAGCAATCC	476	54
ebh	TAAAATAGTAAGCGATAGCG AAGAAAGTACCAGGAGGG	520	54
ebp	AAAAAGACGCAGAGAGACAGC CACCAGCCGCACCAACTCC	560	62
sdrH	TCATTATTTAGTCACCAAGC TGcGAACCCTTACCTGAAC	574	54

were washed once with a sodium chloride irrigation solution 0.9%, and each well was added with 400 µl of 0.1% alcoholic crystal violet solution to stain the formed biofilms.

Staining was performed at 37°C for 1 hour. The crystal violet solution was removed, the wells were washed twice with distilled water, and the crystal violet dye was extracted from the stained biofilm in 200 µl of 96% ethanol for 1 hour at room temperature. As a control, a culture medium was used. The optical density was measured at 570 nm in a Multiskan Ascent 354 Microplate Reader (Thermo Electron Corporation, USA). The measurements were carried out with three biological repetitions. The isolate was considered to be biofilm-forming if the optical density exceeded 0.24 [17].

For SEM, CVC samples were removed from a 10% formalin solution and dried for 10 minutes at room temperature. Before the analysis, all samples were coated with a gold layer with a thickness of 5 nm in an SPI-MODULE Sputter Coater (SPI Supplies, USA). The inner and outer surfaces of the catheters were analyzed using a Quanta 200 3D dual-beam scanning electron microscope (FEI Company, USA) in the high vacuum mode and at a 7.5 kV accelerating voltage.

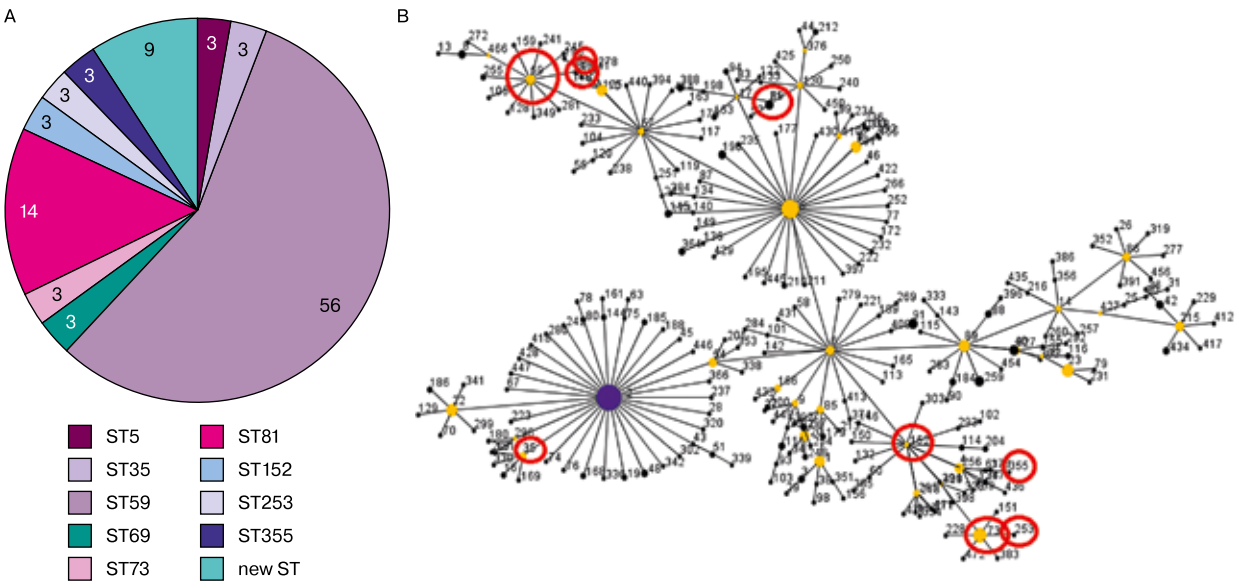
Results and discussion

In the course of the study, a collection of 150 CVCs was analyzed. The bacteriological study result indicated that 53 catheters were colonized by conditional pathogens. Of them, 50 samples were contaminated by a single strain. Most of the isolates were identified as coagulase-negative staphylococci (41/50, 82%), namely *S. epidermidis* (34/50, 68%), which was consistent with the epidemiological studies carried out in other neonatal intensive care units [18]. Apart from coagulase-negative staphylococci, the collection was colonized by single isolates of the following species: *Staphylococcus aureus, Acinetobacter pittii, Aerococcus viridans, Bacillus cereus, Enterobacter cloacae, Enterococcus faecalis, Streptococcus viridans, Pantoea agglomerans, Candida albicans*. Besides, three catheter specimens were colonized by multiple species, specifically: catheter № 47 - *Staphylococcus haemolyticus* and *S. epidermidis*; № 113 - *Staphylococcus haemolyticus* and *Streptococcus* of *viridans* group; № 202 - *S. epidermidis*, *Enterobacter cloacae,* and *Pantoae agglomerans*.

To assess the diversity of *S. epidermidis* isolates from the surface of CVCs, their sequence-types were identified by multilocus sequence typing. The isolates of the collection were assigned to 12 different sequence types, most frequently to ST59 (19 isolates, 56%) (Figure 1A). It is worth noting that nine of identified sequence types belonged to the CC2 clonal complex, which is typical of the hospital acquired *S. epidermidis* (Figure 1B) [12], thus indicating the nosocomial origin of the majority of the collected *S. epidermidis* isolates (31/34, 91%). Besides, among the identified sequence types, there were three new ones with the following allelic profiles: 3-41-5-5-38-5-11; 1-1-1-2-2-1-11; 1-6-1-2-2-1-10. These sequence-types, characterizing three isolates of *S. epidermidis*, do not belong to the CC2 clonal complex.

One of the most important pathogenicity factors for coagulase-negative staphylococci, including *S. epidermidis*, is their ability to form biofilms. Biofilm formed by bacterial cells serves as a protective barrier for them, protecting from antimicrobial agents and natural immune response of the host organism. Most of the isolates (28/50, 54%) had an increased ability to adhere to plastic surfaces (OD> 0.24), and there was no correlation between the belonging to a certain sequence-type and the level of ability to form biofilms. Apart from *S. epidermidis* isolates (n = 23), increased

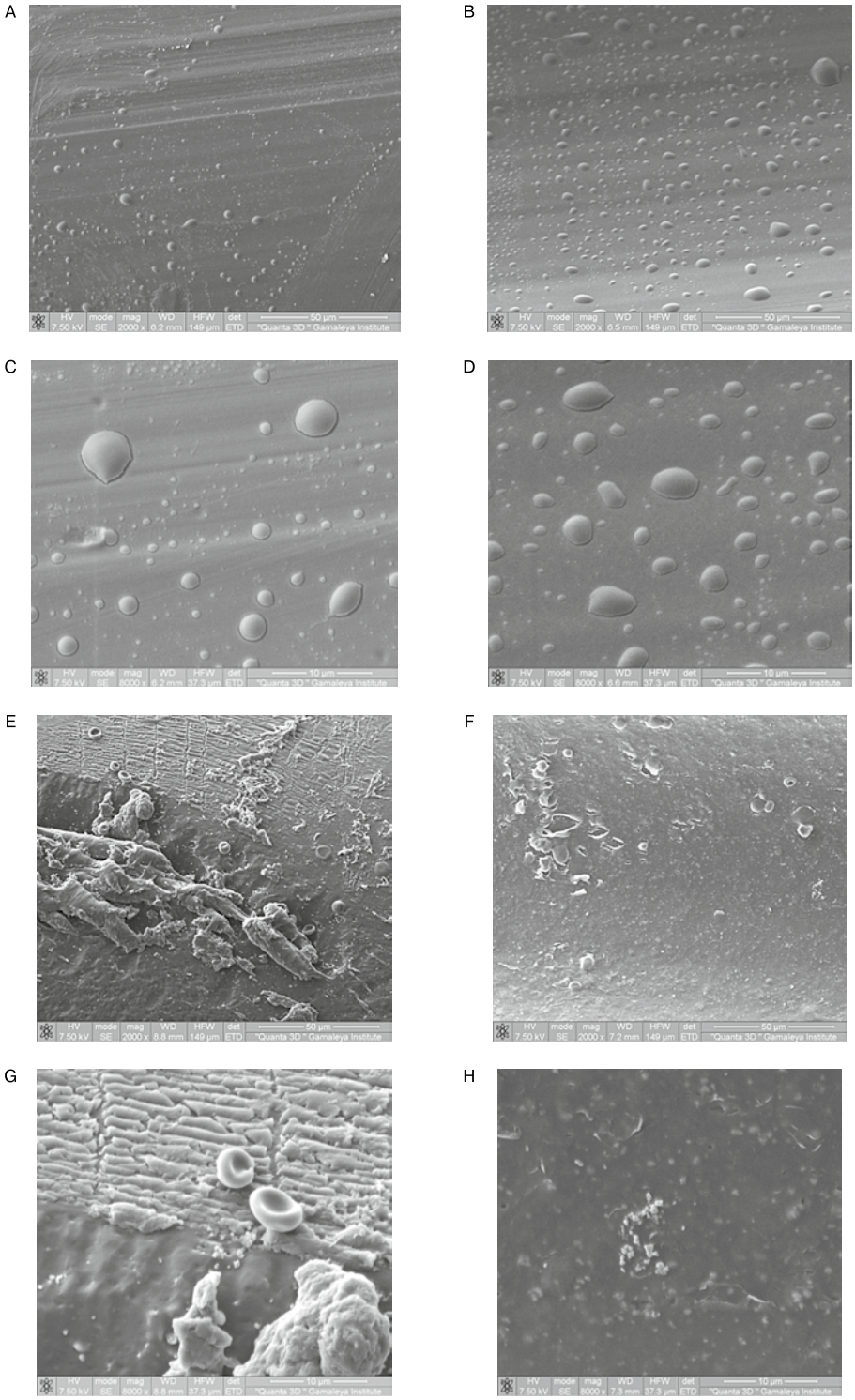
Fig. 1. Multilocus sequencing typing of S.epidermidis isolates: A - Characteristic sequence of S.epidermidis isolates from the surface of CVCs; B - Clonal complex CC2 (sequence types of S.epidermidis isolates from the surface of CVCs marked red)



ability to adhere to plastic was found in the isolates of *Enterococcus faecalis* (n = 1), *Acinetobacter pittii* (n = 1), *S. haemolyticus* (n = 1), *S. warneri* (n = 1), and *Candida albicans* (n = 1). Of note, two isolates of *S. epidermidis* belonging to the new sequence-types (1-1-1-2-2-1-11, 1-6-1-2-2-1-10) also had an increased adherence to plastic surfaces.

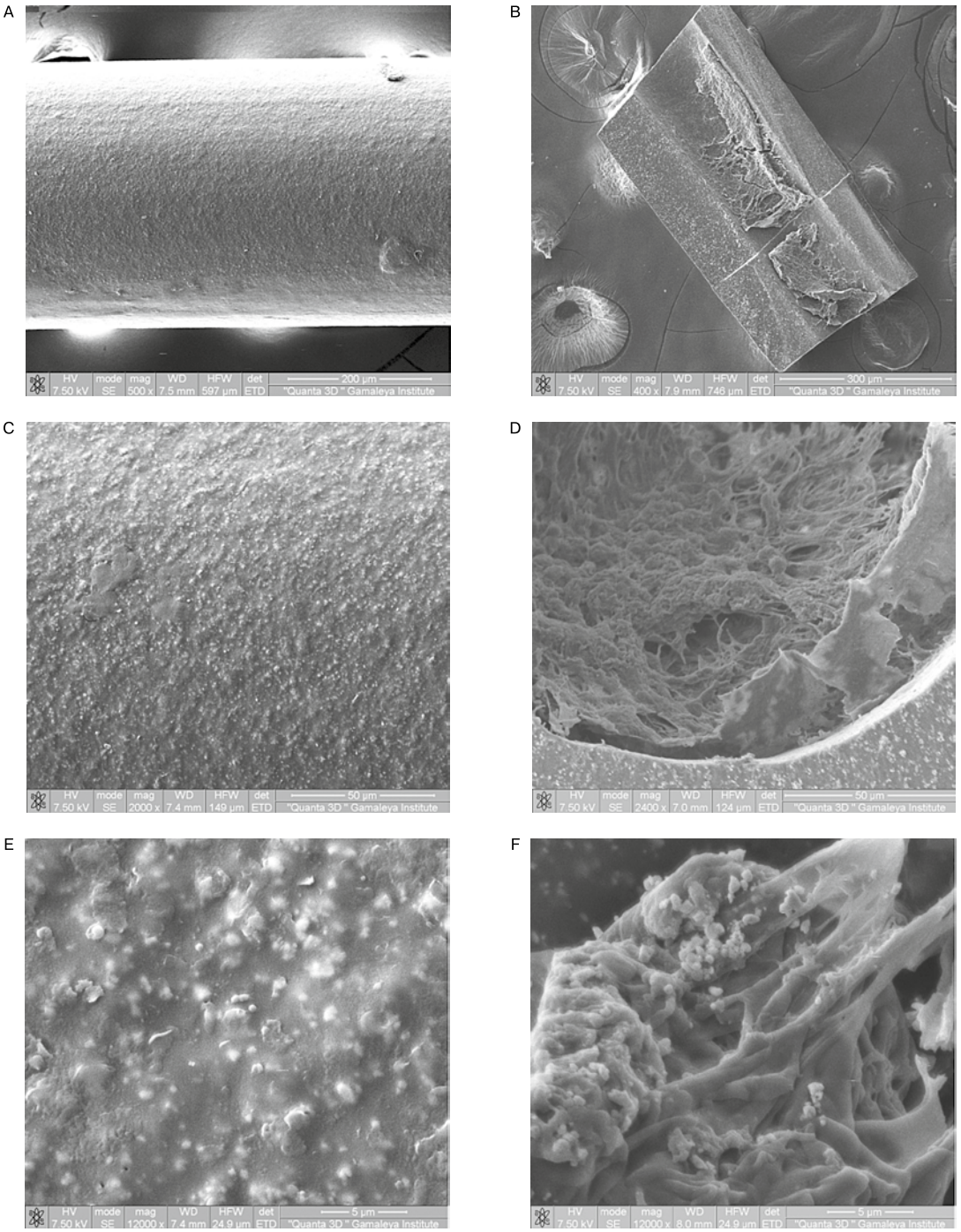
Biofilm formation on the CVC surface was confirmed by SEM of CVC samples *in vivo*. An intact catheter was used as control samples (Figures 2A, B, C, D) and a catheter in a neonate (length of catheterization six

Fig. 2. Scanning electron microscopy of an intact catheter (A, C - outer surface (x2000, x8000), B, D - inner surface (x2000, x8000)) and a catheter used in a newborn for 6 days; culture showed no bacterial growth (E, G - outer surface (x2000, x8000), F, H - inner surface (x2000, x8000))



days) without bacterial growth, according to the culture results (Figure 2 E, F, G, H). SEM results showed a similar structure of the outer and inner surfaces of the intact catheter. In the catheter material, round inclusions (100 nm - 5 μm) were observed (Fig. 2A, B, C, D). On the external surface of the catheter, where no bacterial growth was detected, a significant amount of substance of moderate electron density was visualized (Fig. 2E, G), as well as some red blood cells (Fig. 2G). In turn, the inner surface was covered with less amount of substance of moderate electron density than the outer surface (Fig. 2F, H). Based on the literature data, that the substance of moderate electron density is a human protein representing components of the extracellular matrix, such as collagen, elastin, fibronectin, etc. Neither biofilm formed by bacteria nor individual bacterial cells were detected on the catheter.

Fig. 3. Scanning electron microscopy of a catheter used in a newborn for 4 days; culture yielded a S. epidermidis isolate: A - outer surface (x500); B - inner surface (x 400); C - outer surface (x2000); D - inner surface (x2000); E - outer surface (x12000); F - inner surface (x12000)



The biofilm formed by S. epidermidis is shown in Fig. 3. The length of catheterization was four days. On the external surface of the catheter, a crumb-like material, bacteria and some amount of a homogeneous substance were visualized (Fig. 3A, C, E). There was a segment of the inner surface with a clot on it representing a biofilm consisting of fibrin filaments, blood cells, and bacteria (Fig. 3B, D, F). It should be noted that an isolate grown on this catheter was identified as SE210, characterized by ST59 increased adherence to plastic.

Robust literature demonstrates that the main component of biofilms formed by *S. epidermidis* is the β-1,6-N-acetylglucosamine polysaccharide polymer, also called polysaccharide intercellular adhesin (PIA) [8, 9]. The genes responsible for the PIA synthesis were detected using three different single primer amplification reaction [14-16]. The presence of the icaA, icaB, icaC, icaD genes was confirmed for five isolates of *S. epidermidis* and two isolates of *S. aureus*. The results obtained using different primer systems are completely consistent. The icaABCD operon genes were identified in the isolates of *S. epidermidis*, characterized by sequence types ST73, ST69, ST59, ST35, and the new sequence-type 1-6-1-2-2-1-10. Thus, no icaABCD operon genes were identified in the isolate SE210 obtained from the catheter with the biofilm visualized by SEM. This may be due to the ability of *S. epidermidis* strains to form so-called ica-independent biofilms [19]. In addition to the polysaccharide, the biofilm contains various proteins [8]. The presence of genes of *Staphylococcus aureus* autolysin (AtlA), accumulation-associated protein (Aap), extracellular matrix-binding protein (Ebh), and Ebp and SdrH proteins members of the MSCRAMM family were identified in *S. epidermidis* isolates from the catheter surface. The genes of AtlE and Aap proteins were found in all isolates of *S. epidermidis* (34 isolates, 100%). The *ebh* gene was detected in 21 *S. epidermidis* isolates (21/34, 62%), of which 15 had increased adhesion. As for the genes of the MSCRAMM family, the elastin-binding protein (EBP) gene was found in only seven isolates with increased adhesion (7 / 34, 20%); fibrinogen-binding protein SdrG (sdrG) gene was detected in 29 (29/34, 85%) isolates, including all isolates with increased adhesion.

Conclusion

Therefore, the majority of CVCs obtained after the catheterization of newborns were colonized by *S. epidermidis*. The main pool of epidermal staphylococci isolated from the surface of the catheters belonged to the sequence types within CC2 clonal complex, which confirms the nosocomial origin of these isolates. Based on the results of staining bacterial cells attached to the plastic with crystalline-violet, a group of isolates with an increased ability to adhere to plastics was identified. No correlations were found between the increased capacity of bacterial cells for adhesion to plastic and a certain sequence-type. The differences in the biofilm forming ability of hospital-acquired isolates belonging to CC2 and isolates belonging to a non-hospital population could not be estimated due to small sample size. Thus, the reason why CC2 has an evolutionary advantage in the hospital environment remains unclear.

The ability to form biofilms directly on CVCs was confirmed *in vivo* by SEM. Most isolates did not have icaABCD operon genes responsible for the synthesis of the PIA polysaccharide, while the presence of the genes of proteins involved in biofilm formation was detected in adhesive isolates. Thus, these isolates formed a biofilm on the surface of the catheters via the ica-independent mechanism, which contradicts the concept of the leading role of the icaABCD operon as the main pathogenicity factor associated with biofilm formation. EMS demonstrated on the surface of CVCs recovered from catheterization, there is a significant amount of the substance of moderate electron density, probably representing human cell proteins. Perhaps for the formation of biofilms on the CVC surface with such an amount of extracellular matrix represented by human proteins, bacterial cells do not need to synthesize the PIA polysaccharide and have a sufficient amount of its own protein adhesives (MSCRAMM family proteins) to attach to the surface of the catheter and form the biofilm.

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