ORIGINAL ARTICLE

Pathogenicity Factors of *Kocuria kristinae* Contributing to the Development of Peri-Implant Mucositis

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ABSTRACT

Introduction: The formation of bacterial biofilms by opportunistic microorganisms on the surfaces of the implants is the leading etiopathogenetic factor of peri-implant mucositis and peri-implantitis. The purpose of this research was to study the adhesive and biofilm-forming properties of clinical strains of *K. kristinae* isolated from patients with peri-implant mucositis. **Methods:** 18 clinical strains of *K. kristinae* isolated from the patients were the object of the study. The adhesion of the microorganisms was determined by using formalized human red blood cells of the blood group O (1) Rh+ by the G. Brilis technique. The study of biofilm-forming properties of clinical isolates of *K. kristinae* was performed by using the spectrophotometric technique by G.D. Christensen (MtP microtiter plate test). **Results:** The representatives of genus Kocuria were isolated in 70% of patients. Total proportion of clinical isolates of *Kocuria spp.* among all isolated microorganisms reached 18.3%. The representatives of the species *K. kristinae* showed a high adhesion to red blood cells and high ability to form biofilms. The r-Pearson coefficient (+0.87) between adhesion and the ability to form biofilms by *K. kristinae* strains pointed out a direct correlation. **Conclusions:** Obviously, *Kocuria spp.* take part in peri-implant mucositis development. It is proved by their presence on the mucous membrane of peri-implant area of patients with complications after dental implant placement. The representatives of the species *K. kristinae* showed a high adhesion and high biofilmformation.

Keywords: *Kocuria*, Peri-implant mucositis, Biofilms

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INTRODUCTION

According to the actual data, there are more than 700 species of normal oral microflora, half of which can not be cultivated. In recent studies based on using molecular-biological methods, electron microscopy and bacterial cultivation have suggested that there are some pathogens, which are still unknown or little known that can trigger or contribute to pathogenesis of oral diseases (1).

According to the report of I. Szczerba, bacteria of the genus Kocuria are found on the skin and mucous membranes in 81.3% of the healthy population. Moreover, the microorganisms were more often isolated from the oral cavity (48.7%) (2). Recently, there have been more data indicating the correlation between the opportunistic pathogens *Kocuria spp*. and the development of meningitis, endocarditis, cholecystitis and osteomyelitis in people with immunodeficiencies. Particular attention is paid to the ability of the representatives of this genus to cause bacteremia and sepsis (3-6). There are few articles, that demonstrate role of *Kocuria spp*. in development of pathology of an oral cavity. Maxillary osteomyelitis and black tongue caused by Kocuria species were reported in Turkey (7,8). Moreover, our previous researches have been showing presence of these microorganisms on mucous membrane of oral cavity during complications after dental implant placement and taking part in their development (9). The frequency of Kocuria isolation from inflammatory sites in humans, including oral cavity, and enlargement of case reports about diseases caused by representatives of this genus contribute to careful research of *Kocuria spp*. and their pathogenicity factors.

Undoubtedly, the formation of bacterial biofilms by opportunistic microorganisms on the surfaces of the implants is the leading etiopathogenetic factor of peri-implant mucositis and peri-implantitis. However, non-bacterial factors resulting in the development of complications after dental implantation, e.g. implant breakage or cement leakage under the mucous membrane in the site of dental implant insertion create

the preconditions for the formation of pathogenic microbiota with the subsequent development of infectious and inflammatory processes. Therefore, the thorough investigation of the course of inflammatory processes, which arise around the implants, taking into account the peculiarities of adhesiveness and biofilm formation of their dominant pathogens, contributes to the revision and improvement of already known conservative and surgical methods of the treatment of these diseases as well as to prevention of their development and progression (10-12).

The purpose of this research was to study the adhesive and biofilm-forming properties of clinical strains of *K. kristinae*, isolated from peri-implant mucous membranes in patients with peri-implant mucositis. As their main pathogenicity factors, adhesiveness to mucous membranes and surfaces of implants and biofilm-formation on them set off development of infectious process.

METHODS

The study included 20 middle-aged patients by WHO (mean age was 48 ± 4 years) who had lateral dentition defects and were inserted from 1 to 4 collapsible titanium implants. According to clinical and radiological methods, patients were diagnosed to have peri-implant mucositis. Patients with inflammatory lesions of mucous membrane of oral cavity, signs of peri-implantitis, HIV, diabetes and other chronic diseases in history were excluded from research. Control group includes 10 same-aged patients after collapsible titanium implants placement without any clinical signs of complications. To investigate aerobic and facultative-anaerobic microflora, the material was taken from the peri-implant pocket using a sterile paper endodontic post of standard size (No. 30), 1 cm long followed by cultivation and identification. The final identification was carried out using the automatic bacteriological analyzer Vitec - 2compact bioMérieux (France).

18 clinical strains of *K. kristinae* isolated from the patients examined were the object of the study. The adhesion of the microorganisms was determined by using formalized human red blood cells of the blood group O (1) Rh+ by the G. Brilis technique (13). Adhesive properties were evaluated by the adhesion index (AI) of microorganisms through counting the mean number of microbial cells adhered to one erythrocyte participating in the adhesion process. Adhesion was considered to be null at AI below 1.75, the low level adhesion was at AI ranging from 1.76 to 2.5, the average level was at AI ranging from 2.51 to 4.0, and the high level of adhesion was at adhesion index higher than 4.0.

The study of biofilm-forming properties of clinical isolates of *K. kristinae* was performed by using the spectrophotometric technique by G.D. Christensen (MtP microtiter plate test). Biofilms were reproduced in wells of a sterile, flat-bottom 96-well polystyrene tray (Corning, USA) and stained with 1% solution of crystalline violet. Properties of the microorganisms to form a biofilm were evaluated by the degree of dye absorption in optical density units using a spectrophotometer (570 nm). The ability of microorganisms to form biofilms was assessed as low (at optical density <0.120), average (at optical density = 0.121-0.239) and high (at optical density > 0.240) (14).

The statistical processing of the results obtained was carried out by standard "STATISTICA+" and "Microsoft Excel 2010" software packages. In order to determine the relationship between the adhesion and biofilm formation of *K. kristinae* strains, we determined the correlation coefficient (r-Pearson coefficient), the absolute value of which characterized the binding force.

RESULTS

Among 10 investigated persons, who were include in control group, representatives of Staphylococcus spp. were found in 2 (20%). However, the frequency of occurrence of bacteria from this genera was risen to 90% (Table I).

The results of the study have revealed the etiological significance of the opportunistic pathogens of Kocuria spp., which colonized mucous membranes of the peri-implant site, in the development of peri-implant mucositis. Thus, the representatives of this genus were isolated in 14 (70%) patients, while the frequency of their isolation from control group was 40%.

Table I: The frequency of occurrence of bacteria on human peri-implant mucous membranes in pateints with peri-implant mucositis and control group

MICROORGANISMS	CONTROL GROUP (N=10)		PATIENTS WITH PERI-IMPLANT MUCOSITIS (N=20)	
	Total number	%	Total number	%
Staphylococcus spp.	2	20	18	90
Streptococcus spp.	10	100	17	85
Candida spp.	7	70	17	85
Enterococcus spp.	5	50	8	40
Kocuria spp.	4	40	14	70
Pseudomonas spp.	1	10	8	40
Acinetobacter spp.	1	10	6	30
Esherichia spp.	1	10	4	20

The representatives of the species *K. kristinae* showed a high adhesion to red blood cells, and their microorganism adhesion index equalled 4.11 ± 0.53. At that time, the number of erythrocytes involved into the adhesive process (61.67 ± 7.63%) was quite high and exceeded half of all the erythrocytes investigated (Fig. 1).

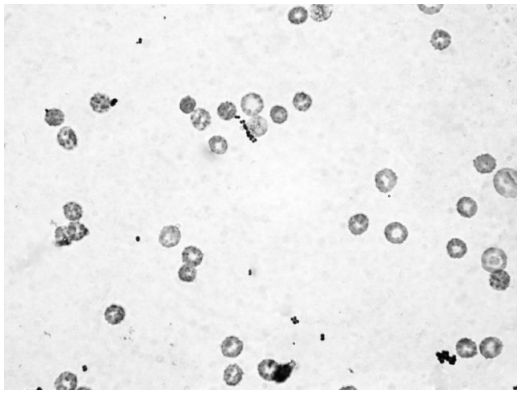

Figure 1: Adhesion of clinical strains of K. Kristinae to human erythrocytes demonstrated on a Gram stained slide (magnification x120)

It has been found out the clinical strains of K. kristinae, colonizing the peri-implant site during peri-implant mucositis, possess high properties to form biofilms. The optical densities of the biofilms formed by these microorganisms were 0.241 ± 0.09 density units for 24 hours and 0.267 ± 0.08 density units for the second day of cultivation.

The r-Pearson coefficient (+0.87) between adhesion and the ability to form biofilms by K. kristinae strains pointed out a direct correlation (Fig. 2). That is, with the increase in microorganism adhesion index of the microorganisms, their ability to form biofilms increases.

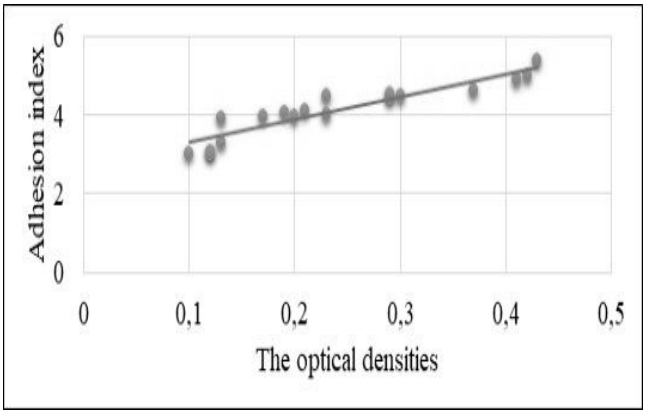

Figure 2: Correlation between microorganism adhesion index and biofilm formation by clinical strains of *K. kristinae* (n = 18).

DISCUSSION

The role of opportunistic microorganisms in the development of infectious diseases is directly related to the presence of pathogenicity factors, and adhesion is one of the most important among them (15, 16). After all, it is the adhesion that initiates the cascade of immunologically mediated reactions determining the specificity of the infectious process, and is its trigger mechanism. Whereas, by overcoming the protective barriers of the macro organism, pathogens interact in specific ways with cells of the mucous membranes, the skin, by attaching to them (17). Infectious inflammatory processes associated with the biofilm formation require special treatment tactics, because the microorganisms within the biofilm have high synergism and represent a highly organized three-dimensional biological form of vital activity of bacteria that can withstand external factors of aggression. An important property of a biofilm is the protection of microorganisms from adverse environmental factors, the action of antimicrobial agents and the factors of the immune system of the macro organism (18).

There are data, that highlight pathogenicity factors and role of *Staphylococcus spp*., *Streptococcus spp*. in the development of dental implant complications, including peri-implant mucositis (19, 20).A.J. Smith and others have summarized that failing dental implants are bound with *S.aureus* solation in 69% of patients. Proportion of *Staphylococcus spp*. in microbiota of affected sites was 15-100% of total flora (21). It is naturally, since those strains' pathogenesis is a direct consequence of the complement of the virulence-associated genes that the organism possesses. Their pathogenesis is multifactorial and includes a comprehensive suite of virulence determinants that includes enterotoxins, quorum sensing mediators, cell lysis mediators, immune system evasion factors, as well as an array of toxins that facilitate tissue destruction. Staphylococcal adherence to an either biotic or abiotic surface is the critical first event in the establishment of an infection with these serious pathogens. Especially *Staphylococcus aureus* harbours a variety of proteinaceous and non-proteinaceous adhesins that mediate attachment to a multitude of host factors, such as extracellular matrix and plasma proteins and human host cells, or intercellular adhesion, which is essential for biofilm accumulation (22,23,24).

However, the prevalence of human infections caused by *Kocuria* species is underestimated, as commonly used phenotypic assays are known to misidentify *Kocuria* isolates as *Staphylococci*. Accordingly, a number of presumed staphylococcal pathologies might have been caused by *Kocuria* species (25). According to the changes in the taxonomy and nomenclature of a bacterium *Kocuria* - a new name of microorganisms previously belonged to *Micrococcus spp.* However, Stackebrand et al. in 1995, based on the phylogenetic

and chemotaxonomic features, isolated the genus *Kocuria* (1,2). Therefore, the lack of definitive data or little data on their biological properties, and, as a consequence, on the approaches to the treatment of infections caused by *Kocuria spp*. is quite explainable.

Biofilm formation of Kocuria starts from the colonization of the tissues by them via hydrophobic, electrostatic and Van der Waals forces. Moreover, teichoic acids, which are in structure of all Gram-positive bacteria, take part in adhesion to substrate. Because of being a foreign material, implant is covered by soluble human proteins like collagen, fibronectin, albumin etc. Attachment of *Kocuria spp*. to them is provided by microbial surface components recognising adhesive matrix molecules (26). A high index of adhesiveness is quite typical for the representatives of *Kocuria spp*., that explains their role in beginning of biofilm formation. The second stage of biofilm development is realized after adhesion and characterized by multiplication of microorganisms and formation of cell clusters. This proved that biofilm formation depends on adhesiveness of bacteria and there is direct correlation between those processes.

CONCLUSIONS

The data obtained provide an insight into properties of *Kocuria spp*. and their role in the development of plaque-associated diseases of the oral cavity, including peri-implant mucositis, as well as contribute to re-thinking the tactics of their prevention and treatment, taking into account the features of the dominant microflora. Consequently, physicians have to count susceptibility of peri-implant microbiota to commonly used chemotherapeutic agents and implement new antiseptics which have activity against adhesiveness and biofilm formation of pathogens causing mucositis.

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