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Bacteria Forming a Resident Flora of the Skin as a Potential Source of Opportunistic Infections

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A b s t r a c t

Along with progress of medicine, contribution that opportunistic bacteria make in nosocomial infections increases. Coagulase-negative staphylococci are these multiresistant strains which often cause this kind of infections. But more and more frequently other genera of bacteria are isolated. The main source of them is first and foremost the hospitalized patient's endogenous flora *e.g.* from their skin, because transmission of bacteria from this source is very effective. Analysis was concerned with bacteria that were recovered repeatedly from the skin of young, healthy men during period of five months. Composition of resident bacteria, after removing transients was evaluated. The number of microorganisms per 1 cm^2 patients' skin was a constant value but different for each patient. Newly composed media enabled exact isolation and qualitative analysis of all groups of expected bacteria. Isolated microorganisms represented three main groups: sensitive to novobiocin staphylococci, microaerophilic rods from *Propionibacterium* genus and coryneform bacteria. Aside from quantitative differences in total bacteria number, significant differences in contribution of aerobic and anaerobic flora living on patient skin were observed. A persistent although not predominant population occurring on the skin of all patients in similar number (average 2%), were coryneform bacteria. They mainly belonged to the *Corynebacterium* genus, and 84.7% of them were the lipophilic species. These bacteria deserve special attention because among such species isolated from nosocomial infections, multiple antibiotic resistance of unknown origin was described.

K e y w o r d s: resident flora, human skin

Introduction

The role of coagulase-negative staphylococci as opportunistic pathogens is well-known. They cause serious nosocomial infections in immunocompromised patients and in patients who underwent invasive diagnostic tests and invasive medical treatment, especially with using implants and foreign bodies (von Eiff *et al*., 2001; 2002; Rupp and Archer, 1994). The skin is a source of those microorganisms, they are an important component of its normal flora. A microorganism selection, which takes place as a result of antibacterial drugs administration leads to the development of multiresistant hospital strains. Besides staphylococci, the human skin is colonized by a wide variety of other microorganisms. The objective of the current study was an analysis of human skin microbial flora in order to find microorganisms which, as resident flora, could represent similar danger of becoming a source of opportunistic infections as coagulase-negative staphylococci.

Experimental

Materials and Methods

Samples. Samples were collected from the skin surface of five young men aged 24–26. They were healthy individuals without any visible pathological changes on their skin. Two sites were sampled: the forehead above eyebrow ridges and the back, between shoulder blades. Samples were taken three times at a few weeks intervals (4–6 weeks); in the period from January to June, in the

morning. Patients were requested not to wash these places of their bodies in these mornings. To the area from which a sample was taken, a sterile, warm (50°C) compress moistened 0.85% NaCl supplemented with 0.2% Tween 80 (Sigma) was stuck. After two minutes compress was removed. From the area of 20 cm^2 swabs were taken with sterile gauze moistened with 0.85% NaCl with 0.2% Tween 80.

Cultures. Microorganisms from the swabs were eluted by shaking in 1.9 mL 0.85% NaCl with 0.2% Tween 80. Suspension was diluted 10^4 in serial dilution method. Every dilution (0,1 mL) was inoculated on solid media: TYT80 [Tryptic Soy Agar (Graso) supplemented with 0.3% Yeast Extract (Difco), 0.05% Tween 80 (Sigma) and 5% (v/v) defibrinated sheep blood] – basic medium constructed for recovering of all bacteria growing in an aerobic atmosphere; TYT80-BAC [medium TYT80 supplemented with 0.3 μg/mL bacitracin (MERCK)] – selective medium for isolation gram-negative flora of the skin; TYT80-MUP-[medium TYT80 supplemented with 2 μg/mL mupirocin (SmithKline Beecham Pharmaceuticals)] – selective medium for isolation of gram-positive rods and micrococci and VL-MUP medium consisted of: Beef Extract (Difco) 0,3%, Pepton Proteose (Difco) 1% (w/v), Yeast Extract (Difco) 0.5%, cysteine hydrochloride 0.04%, agar 2% (w/v), 1 mL/100 mL solution of hemin (0.05%) and vitamin K (0.001%), 5% (v/v) defibrinated sheep blood according to Burbianka and Pliszka (1977) supplemented with 2 μg/mL mupirocin – selective medium for isolation of anaerobic bacteria resistant to mupirocin. Cultures on TYT80 medium were incubated in ambient air, at 37°C for 72 hours, and the next 48 hours at 25°C exposed to the light (Kloos *et al.*, 1991). Cultures on VL-MUP medium were incubated in an anaerobic jar with Generbox Microaer (bioMerieux) for five days. Afterwards colonies were counted and isolated adequately to basic TYT80 medium or VL medium without antibiotic for further identification.

Identification. Gram-positive, irregularly shaped rods were additionally stained according to Neisser method to detect the presence of metachromatic granules. All isolates were tested for catalase. Gram-positive cocci were differentiated on the basis of their furazolidone sensitivity [Mueller-Hinton Agar 2 (bioMerieux) with 2 mg/mL furazolidone]. Further identification was conducted to divide staphylococci isolates into the groups according to Shleifer *et al.* scheme (Schleifer and Kroppenstedt, 1990). Catalase-positive, gram-positive, irregular shaped rods, grown in ambient air were tested for lipid requirements with the use of broth medium BHI (Difco) supplemented with 1% (v/v) Tween 80. The isolates were incubated for 120 hours and their growth was estimated every 24 hours in comparison to the cultures of the same isolates on the medium without this substrate. Microorganisms, which demonstrated equally abundant growth in both test tubes, were recognized as nonlipophilic. Isolates, which growth was essentially increased by Tween 80, were recognized as lipophilic (Koneman *et al.*, 1997). Colonies from VL-MUP medium were grown parallely in two cultures in ambient air and in microaerophilic atmosphere in order to distinguish facultative anaerobes from microaerophilic bacteria. Microaerophilic, catalase-positive, gram-positive rods were identified through the biochemical tests. Liquid VL media supplemented with 1% (w/v) glucose, maltose, sucrose, glycerol or tryptophan were used.

T-test for independent samples was applied with use of the computer program Statistica 5.0 StatSoft.

Results and Discussion

A wide documentation concerned flora of the skin, points out the coagulase-negative staphylococci as the most abundant microorganisms of skin flora (Roth and James, 1989; Mackowiak, 1982). Due to easy and frequent isolation of these microorganisms, sometimes they are wrongly recognized as the main element of skin flora (Roth and James, 1989; Mackowiak, 1982). Wilburg *et al*. (1981; 1984) pointed to other also important components: *Propionibacterium acnes, Micrococcus* spp., *Corynebacterium* spp., *Corynebacterium* "*lipophylicum*", and also less numerous *Enterobacteriaceae* and other bacteria. Roth and James (1989) reported participation of coagulase-negative staphylococci, *Micrococcus* spp., coryneform organisms (*Corynebacterium, Brevibacterium*), *Propionibacterium* and *Acinetobacter.*

Kloos and Lambe (1991) took note of the fact that individual species of staphylococci**,** isolated from the skin, showed a preference for the specific regions of the body. The most varied staphylococcal flora occurrs on the face and the forearms. In the author's opinion, quantitative relations between species depend strictly on the age of the examined people. Other authors mention (Roth and James, 1989; Wilburg, 1981; Wilburg *et al*., 1984). a similar phenomenon concerning the other components of skin flora. They also subject the number and composition of skin flora to sex.

Presented research was based on analysis of samples taken from a very homogenic group of mature, young males. Swabs were taken from two different places (forehead and back), where numerous sebaceous glands occurred. Because of the difficult access, the skin between shoulder blades is poorly subjected to an action of cleansing agents, although probably heavily rinsed with water during hygienic procedures. While establishing technique of taking research material, it was assumed that samples should include mainly residential flora of the skin. Warm, damp compresses were used to remove from the skin the majority of transient flora and transient residents, which lived too short on the skin to colonize its deeper layers.

It was expected that a significant percentage of gram-positive cocci and a relatively lower percentage of gram-positive rods would be observed in the taken material (Wilburg, 1981). Consequently, four special rich media were made to ensure isolation of the highest number and variety of microorganisms. All of them were examined by means of culture of adequate suspensions of reference strains with density comparable to the tested samples (*S. aureus* ATCC 25923, *S. epidermidis* CCM 2354, *Corynebacterium diphteriae* biotype mitis PCM 506, *E. coli* ATCC 10538, *Propionibacterium acnes* 227).

Basic medium TYT80 was formulated for the recovery of all aerobic and facultative anaerobic bacteria from the skin swabs. Particular attention was paid to the isolation of coryneform bacteria. Basic medium along with nutrients included Tween 80, which increased growth of lipophilic bacteria and did not slow down growth of the other bacteria. In order to construct the selective medium for coryneforms (TYT80-MUP), their natural resistance to mupirocin was used. It is known that their growth is not inhibited in the presence of the antibiotic (MIC > 128 μg/mL) while staphylococci are naturally sensitive to it (MIC < 0.5 μg/mL) (Hryniewicz and Meszaros, 2001). Consequently the growth of dominant part of bacteria from the samples on this medium was inhibited. Mupirocin was also a factor in selecting of resistant to this antibiotic microaerophile rods on VL-MUP medium. On media without this antibiotic, also in a microaerophilic atmosphere, staphylococci grew heavily and suppressed growth of the other species. Acquired resistance to mupirocin sometimes occurs in *Staphylococcus* strains selected in a consequence of contact with this antibiotic. This kind of strains relatively easily aquire this resistance, but also easily lose it. Upon examination our patients declared that they had not been exposed to any antibiotic treatment and had not used any ointment containing mupirocin in recent months. Few of staphylococci colonies which grew on medium with mupirocin clearly differed from naturally resistant coryneform bacteria and single micrococci. Inoculation of low dilution of samples enables the isolation of rare species of skin flora. Conditions needed to grow gram-negative bacteria were created by TYT80-BAC medium containing bacitracin, which inhibited gram-positive flora from growing.

Colonies grown on all kind of media were counted and their number was compared and balanced. First results showed that each patient should be considered individually. Table I shows the total number of bacteria per 1 cm^2 skin of the back and forehead of patients. These are results obtained from the samples taken in three turns, at intervals of few weeks. The number of bacteria per 1 cm^2 on the skin of the back varied from 5×10^3 to 62×10^3. For the skin of the forehead this number was lower – 3×10^3 – 23×10^3. The smallest number of bacteria in both examined places was isolated from person A and the biggest from person R. The numbers of bacteria in all turns were similar and the biggest standard deviation concerned results of samples taken from person A. However, a clear diversity of mean values can be seen between individuals. It can be assumed that the number of all bacteria per $1cm^2$ of the back skin and the forehead skin is an individual, constant and time-persisted feature. Results regarding samples from the forehead and the back were compared statistically. Gathered results of all the samples taken from all five patients, separately for the back and the forehead, had a parametric distribution, therefore test-t for independent samples was used. Statistical analysis showed that the difference between average numbers of bacteria per 1 cm^2 of the back skin and the forehead skin is statistically significant ($p<0.016$).

Table I
Total number of bacteria per $1cm^2$ of skin (cfu × $10^3/1cm^2$)

Sample	Number of bacteria (cfu × 10^3)									
	Back					Forehead				
	A	B	K	P	R	A	B	K	P	R
1	2.72	49.75	7.49	25.92	62.33	3.50	11.90	10.83	16.31	22.35
2	7.23	42.26	11.94	28.66	82.64	4.12	22.74	11.99	16.22	23.04
3	4.29	50.45	11.22	26.81	41.24	1.76	13.04	8.27	16.18	23.34
Mean	4.75	47.49	10.22	27.13	62.07	3.13	15.89	10.36	16.24	22.91
Standard deviation	2.29	4.54	2.39	1.40	20.70	1.22	5.96	1.91	0.07	0.51

Table II shows average numbers of bacteria on the forehead and the back skin in comparison with data obtained through interviews with patients and observation of the skin in examined places. It also shows a percentage of dominant groups of isolates in samples: grown in ambient air (aerobes) bacteria or microaerophiles. Perhaps the smallest number of bacteria in the sample from the skin of person A is connected with the application of antibacterial soap, but on the other hand the application of antibacterial face gel by person K had no impact on the number of the skin residents. We should consider cleansing agents of this kind have a clear effect mostly on a transient flora. Features of the skin of examined people do not seem to have a connection with the number and profile of bacteria isolated from their skin.

Table II
Some features of the skin of examined patients

Forehead	Average number of bacteria (cfu × 10^3/1cm^2)	Dominant flora	Percentage of coryneform bacteria	Sweating of the skin	Oily skin	Use of antibacterial soap	Tendency to comedos
A	3.1	a – 97.8%	3.2%	–	–	+	+/–
B	15.9	m – 88.6%	0.5%	+	+	–	–
K	10.4	m – 66.5%	0.7%	+/–	+	+	+
P	16.2	m – 95.7%	0.2%	+	–	–	–
R	22.9	m – 89.2%	0.6%	+/–	–	–	+

Back	Average number of bacteria (cfu × 10^3/1cm^2)	Dominant flora	Percentage of coryneform bacteria	Sweating of the skin	Oily skin	Use of antibacterial soap	Tendency to comedos
A	4.8	a – 99.3%	3.1%	–	–	+	–
B	47.5	a – 97.5%	11.0%	+	+	–	–
K	10.2	m – 93.8%	0.2%	+	+	–	+/–
P	27.1	m – 58.3%	0.2%	+	–	–	–
R	62.1	m – 97.8%	0.2%	+	+/–	–	–

m – microaerophiles; a – aerobes

Fig. 1 shows a percentage of both bacteria grown in ambient air (aerobes) and microaerophiles in population from the skin of the back and from the forehead. The average results were showed. These results were characterized by high repeatability. Meanwhile**,** high diversity of the results among patients was observed. With regard to a little importance of calculating mean values for all five patients in further analyses all results were considered individually for every single patient.

While analyzing these graphs it can be observed that an average percentage of aerobic and microaerophilic bacteria isolated from every single patient differs significantly, even between the forehead and the back of the same person.

An attempt of identifying all isolates was made. It was assumed that diversity of population represented in dilutions 10^2–10^4 corresponded with diversity of population of an undiluted sample. The isolation of colonies from all applied media allowed to separate 1000 isolates of gram-positive cocci, 485 isolates of gram-positive rods grown in ambient air and 103 isolates of gram-positive microaerophile rods. The majority of isolated gram-positive cocci (97.2%) belonged to *Staphylococcus* genus (a typical arrangement of cells in a microscope, a positive catalase test, sensitivity to furazolidone). Among them 97.7% were

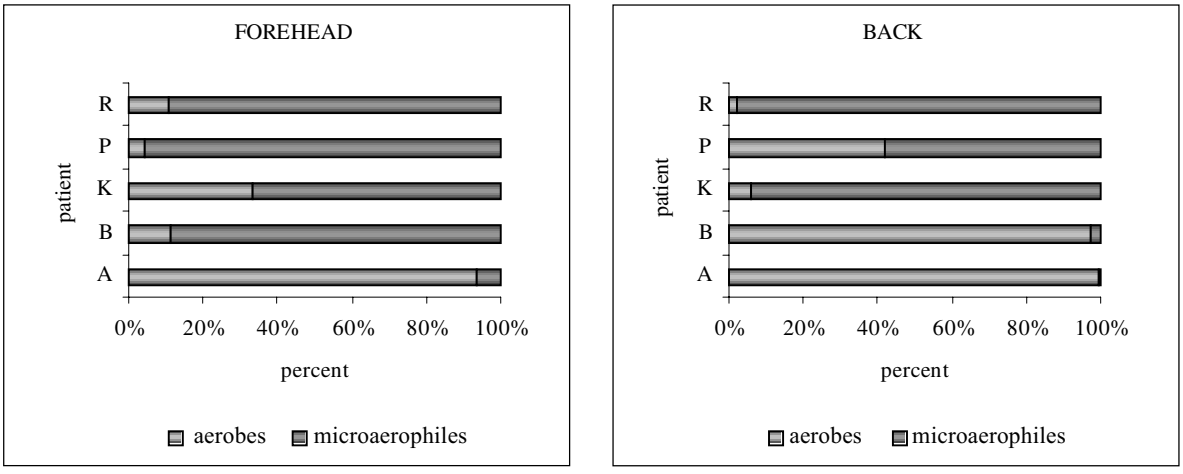

Fig. 1. Percentage of aerobes and microaerophiles in bacteria population of the skin of the forehead and back.

sensitive to novobiocin. It allows to presume that most of them belonged to staphylococci species placed in S. epidermidis and S. simulans groups.

Gram-positive, catalase-positive irregularly shaped rods grown in ambient air were specified as coryneform bacteria (Funke *et al*., 1997). Taking into account the features mentioned above and the presence of metachromatic granules in their cells, the majority of them may be classified as *Corynebacterium*. The lipid requirements showed that most of isolates were lipophilic (84.7%). All isolates, grown only in limited oxygen access conditions (microaerophiles) were catalase producers. They decomposed glucose and glycerol but not maltose and sucrose. Since they also produced indol from tryptophan, they were classified as *Propionibacterium* genus.

Some authors locate propionibacteria in the coryneform group (Funke *et al*., 1997) but more frequently this name refers only to aerobic rods-diphteroids.

On the base of these results all isolates from the skin that were found to belong to three main groups of microorganism were determined as novobiocin-sensitive staphylococci, coryneform bacteria and microaerophile rods-genus *Propionibacterium*. Figure 2 shows a percentage of bacteria belonging to these groups in samples from the forehead and back. Significant differences between samples isolated from the back and the forehead, even in these obtained from the same patient, were observed.

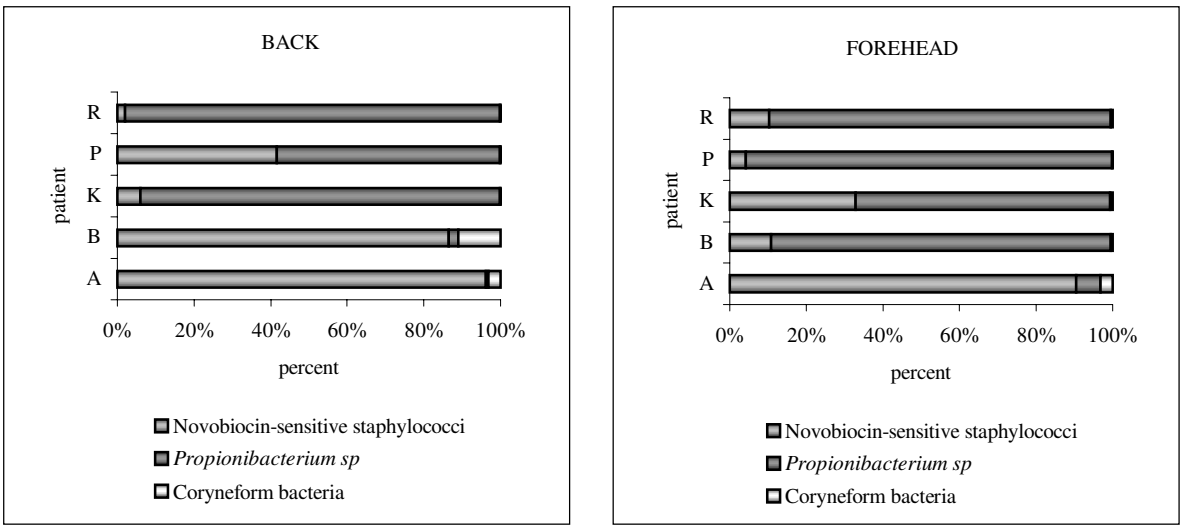

Fig. 2. Percentage of novobiocin-sensitive staphylococci, *Propionibacterium* spp. and coryneform bacteria in population of skin of forehead and back.

While analyzing these figures it can be observed that the distribution of novobiocin-sensitive staphylococci and propionibacteria in patients, skin flora differed significantly. A percentage of coryneform bacteria in samples from the skin in particular patients was relatively small for all patients, although it was slightly higher in the samples from the back of person A and B.

In spite of differences in total number of bacteria living as residents on 1 cm^2 of the skin between the samples from the forehead and the back and insignificant fluctuations between series of samples, it should be pointed out that a percentage of bacteria of each group in the same skin area and from the same person was a constant value. Samples were collected over a period of five months and observed deviations could be connected with seasonal changes and consequently temperature, humidity and type of worn clothes. The fluctuations in number of bacteria were small, and it can be observed that quantities as well as mutual relations between microorganisms were constant and characteristic for every patient.

Although aerobic gram-positive rods formed the smallest group of bacteria, they were the only component that appeared in relatively constant number in every sample taken. Table III shows their participation in the three dominated groups in every turn of samples. The results show high repeatability.

Lipophilic isolates dominated in coryneform group and this observation confirmed results obtained by Roth and James (1989). However, Wilburg *et al*. (1981) in their publication pointed out domination of nonlipophilic species among sparsely isolated representatives of *Corynebacterium* genus. It should be

Table III
Groups of bacteria in every sample

FOREHEAD	A			B			K			P			R		
	Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria	
1	3.5	NS	91.0%	11.9	NS	14.9%	10.8	NS	35.3%	16.3	NS	4.8%	22.4	NS	11.2%
		CB	4.0%		CB	1.0%		CB	1.7%		CB	0.2%		CB	0.7%
		MR	4.9%		MR	84.0%		MR	62.9%		MR	94.8%		MR	88.0%
2	4.1	NS	92.5%	22.7	NS	7.6%	12	NS	36.0%	16.2	NS	1.8%	23	NS	9.7%
		CB	2.6%		CB	0.1%		CB	0.3%		CB	0.2%		CB	0.3%
		MR	4.7%		MR	92.1%		MR	63.6%		MR	97.8%		MR	89.9%
3	1.8	NS	87.7%	13	NS	10.1%	8.3	NS	27.3%	16.2	NS	5.9%	23.3	NS	9.9%
		CB	3.1%		CB	0.4%		CB	0.1%		CB	0.2%		CB	0.8%
		MR	9.1%		MR	89.4%		MR	72.5%		MR	93.8%		MR	89.1%

BACK	A			B			K			P			R		
	Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria		Total number cfu × 10^3/1cm^2	Groups of bacteria	
1	2.7	NS	97.5%	49.8	NS	90.1%	7.5	NS	3.7%	25.9	NS	52.3%	62.3	NS	2.2%
		CB	1.7%		CB	7.4%		CB	0.2%		CB	0.2%		CB	0.3%
		MR	0.8%		MR	2.3%		MR	96.0%		MR	47.5%		MR	97.4%
2	7.2	NS	96.6%	42.3	NS	84.3%	12	NS	2.1%	28.7	NS	31.7%	82.6	NS	0.7%
		CB	2.5%		CB	11.9%		CB	0.1%		CB	0.2%		CB	0.1%
		MR	0.9%		MR	3.7%		MR	97.6%		MR	68.0%		MR	99.1%
3	4.3	NS	94.3%	50.5	NS	84.9%	11.2	NS	12.1%	26.8	NS	40.5%	41.2	NS	3.1%
		CB	5.3%		CB	13.5%		CB	0.4%		CB	0.1%		CB	0.3%
		MR	0.4%		MR	1.5%		MR	87.4%		MR	59.4%		MR	96.5%

NS – Novobiocin-sensitive staphylococci; CB – Coryneform bacteria; MR – Microaerophilic rods

noticed, however that these authors analyzed both transient and resident flora of skin. It is likely that lipophilic species find better conditions for their existence in deeper layers of epidermis.

Table IV shows a percentage of lipophilic *Corynebacterium* spp. in coryneform bacteria group in all examined samples. The mean standard deviation amounted 16% of the mean value for all taken samples. The results showed that they were common, constant components in population of bacteria, which are residents of the skin.

The opportunistic pathogenicity of lipophilic species of *Corynebacterium* has already been described in the seventies. Isolation of these species was connected with the number of infections, septicemias, especially in patients with implants. Strains of *C. jeikeium* isolated from these clinical cases were resistant to a large number of antibiotics. Infections caused by *C. urealyticum* concerned mainly the urinary system. The latest literature describes infections caused also by another lipophilic species of *Corynebacterium: C. afermentans* subsp *lipophilum* (Sewell *et al*., 1995), *C. accolens* (Claeyes *et al*., 1996), *C. kroppenstedtii* (Bernard *et al*., 2002). The fact is emphasized that under occlusive dressings of wounds favourable conditions are created for the growth of coryneform bacteria. Roth and James (1989) described that in such conditions the number of these bacteria increased significantly and they could outnumber cocci. Inevitably, a change in these relations leads to the development of wound infections caused by corynebacteria. These reasons

Table IV
Percentage of lipophilic species of *Corynebacterium* among all isolated coryneform bacteria

Collection	Forehead	Mean	Standard deviation	Back	Mean	Standard deviation
1A	82.1%	77.2%	6.7	68.4%	79.1%	9.4
2A	69.6%			83.3%		
3A	80%			85.7%		
1B	98.2%	91.4%	13.4	100%	87.0%	22.5
2B	100%			100%		
3B	76%			61.1%		
1K	94.1%	88.0%	15.9	100%	89.7%	17.8
2K	70%			69.2%		
3K	100%			100%		
1P	100%	89.7%	11.8	100%	83.3%	18.4
2P	92.3%			86.4%		
3P	76.9%			63.6%		
1R	82.4%	85.2%	13.6	71.4%	76.6%	6.1
2R	73.3%			83.3%		
3R	100%			75%		

and incomplete knowledge concerning taxonomy and characteristics of this group of bacteria determinate the direction of further research.

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