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Opportunistic Coryneform Organisms – Residents of Human Skin

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A b s t r a c t

Opportunistic infections are usually caused by endogenic flora originated from physiological flora. In this context we studied coryneform bacteria recovered from deeper layers of epidermis of the forehead (278 isolates) and the back (196 isolates) of healthy men. It was observed that coryneform bacteria are in dynamic equilibrium with coagulase-negative staphylococci and they amount 4,7% of resident aerobic flora. On the base of biological and metabolic features 49 different biotypes were indicated. Biotypes of lipophilic rods were the basic part of coryneform flora existing for long period. The most frequently isolated taxa were *C. jeikeium* (31%), CDC group G2 (23,4%), next in order CDC group G1 (11%) and *C. afermentans* ssp. *lipophilum* (7%). These isolates were phenotypically differentiated. Nonlipophilic species did not play significant role in creating resident flora of the skin. The significance of coryneform bacteria in opportunistic infections is growing, especially in immunocompromised patients. Isolated lipophilic taxa belong to these taxa coryneforms which are described in literature as the main etiologic factors of opportunistic infections.

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

Introduction

In clinical laboratory the term "coryneform bacteria" is used for aerobically growing, asporogenous, non-partially acid fast, irregulary shaped gram-positive rods. The diverse genera that have been included within coryneform groups involve for instance *Corynebacterium*, *Dermabacter*, *Brevibacterium*, *Actinomyces*, *Arcanobacterium*, *Turicella* and other numerous pleomorphic rods (Coyle and Lipsky, 1990; Funke *et al*., 1997). The contribution of coryneform rods to opportunistic infections, including nosocomial infections, is still growing. Till recently the presence of these bacteria in clinical samples was associated only with the contamination from skin surface and mucous membranes. However, the growing number of publications dealing with the coryneform's contribution to the infections made us pay attention to this group of bacteria. There is strong need for describing this not very well known group of bacteria. There is little known about their habitats. Their taxonomy is highly unsatisfactory. In such situation it is hard to estimate which species of coryneform should be considered more thoroughly. It is also necessary to improve methods of isolation and identification of these bacteria. Skin is a very important source of microorganisms responsible for the opportunistic infections. One of the most significant example are coagulase-negative staphylococci that are the main cause of nosocomial infections (Rupp and Archer, 1994; von Eiff, Proctor and Peters, 2001; von Eiff, Peters and Heilmann, 2002). Many authors report on the infections caused by other microorganisms connected with the skin surface and mucous membranes, including coryneform bacteria (Coyle and Lipsky, 1990; Funke *et al*., 1997; Bernard *et al*., 2002). In our previous publication (Kaźmierczak and Szewczyk, 2004) we have proven that coryneform are the common, constant, although rather small, component in the population of skin residents. That publication aimed at analysis of a number and frequency of coryneform in the flora of the skin, the composition of their population and their relations with the coagulase-negative staphylococci (CNS) living in the same habitat.

Experimental

Materials and Methods

In this research 474 isolates of gram-positive, aerobic or anaerobic catalase-positive rods recovered from the skin of healthy men – 278 samples from the skin of the forehead and 196 from the skin of the back – were studied. The ratio of the number of these rods to the number of coagulase-negative staphylococci in every sample was counted according to the methods described in our previous publication (Kaźmierczak and Szewczyk, 2004). Most of the isolates were originated from colonies growing on the TYT80-MUP medium that had been constructed specifically for the isolation of the group of these microorganisms (Kaźmierczak and Szewczyk, 2004). Single isolates were originated from the TYT80 medium. Lipophilism of these isolates has been researched earlier (Kaźmierczak and Szewczyk, 2004).

Test for lipophilism allows divide the isolates into two groups: 406 lipophilic isolates and 68 nonlipophilic ones. Differentiation inside the groups was made on the basis of biological and biochemical tests. Acid production from carbohydrate was determined on the liquid medium G-P (according to API-Coryne – BioMerieux) with addition of 1% of examined carbohydrate (glucose, maltose, sucrose, mannitol, lactose). For the lipophilic isolates, medium was enriched by the addition of the inactivated horse serum (3%). Results were being observed within the period of 2 weeks. The esculin hydrolysis was examined on the Enterococcosel Agar Medium (Becton Dickinson). Ability to reduce nitrate to nitrite was determined on the Nitrate Broth (Difco) medium with addition of 1% of Tween 80 (Sigma). Urease was detected on the Christensen's medium with addition of 1% of Tween 80. Test CAMP (Christie-Atkins-Munch-Peterson) was made on BHI (Graso) medium with addition of 5% of sheep blood. Reference strain *Staphylococcus aureus* ATCC 25923 was used for testing. Microscopic characteristic of cells, the features of colonies and the intensity and hemolysis markings in CAMP test were considered as additional features in the classification of the isolates. Differentiation of the isolates was made according to the schema worked out on the basis of the given literature (Sneath *et al*., 1986; Coyle and Lipsky, 1990; Holt *et al.*, 1994; Koneman *et al.*, 1997; Funke *et al.*, 1997, 1997a, 1998; Funke *et al.*, 1997b; Zimmermann *et al.*, 1998; Rassoulian *et al.*, 2001; Renaud *et al.*, 2001; Bernard *et al.*, 2002; Shukla *et al.*, 2003). Schema comprised the identification of bacteria classified as coryneform connected with human: residents of the skin surface and mucous membranes or these of clinical importance. Five genera of bacteria were considered: *Corynebacterium* (29 species and 3 CDC groups), *Brevibacterium* (2 species), *Dermabacter* (1 species), *Actinomyces* (2 species), *Turicella* (2 species).

Results

As it had been already shown, the quantity physiological flora of the skin is specifically differentiated (Roth and James, 1989; Kaźmierczak and Szewczyk, 2004). Also the composition of the flora of every patient differs as to the ratio of aerobic flora to anaerobic one (Kaźmierczak and Szewczyk, 2004). The former consisted mainly of coagulase-negative novobiocin-sensitive staphylococci (CNS) and coryneform bacteria. Their number for the 1 cm^2 of skin surface depends on the person (A, B, K, P, R – see Table I). There were also some differences between flora of the back skin and the forehead skin of the same person. However, it appeared that ratio between the number of CNS and coryneform rods is a constant value (95.3% to 4.7%) (Fig. 1).

Table I
Coryneform bacteria and coagulase negative staphylococci (CNS) in aerobic flora of human skin

Back		Coryneform bacteria cfu/1 cm^2	CNS cfu/1cm^2	% coryneform bacteria	% CNS
person	A	150.3	4563.0	3.2	96.8
	B	5183.3	41150.0	11.2	88.8
	K	19.0	631.0	2.8	97.2
	P	42.3	11174.3	0.4	99.6
	R	118.7	1086.3	8.9	91.1
mean				5.3 ± 4.6	94.7 ± 4.6
Forehead		Coryneform bacteria cfu/1 cm^2	CNS cfu/1cm^2	% coryneform bacteria	% CNS
person	A	100.6	2849.4	3.5	96.5
	B	67.4	1601.6	4.0	96.0
	K	71.6	3465.1	1.8	98.2
	P	31.5	671.8	5.9	94.1
	R	133.4	2349.9	5.3	94.7
mean				4.1 ± 1.6	95.9 ± 1.6

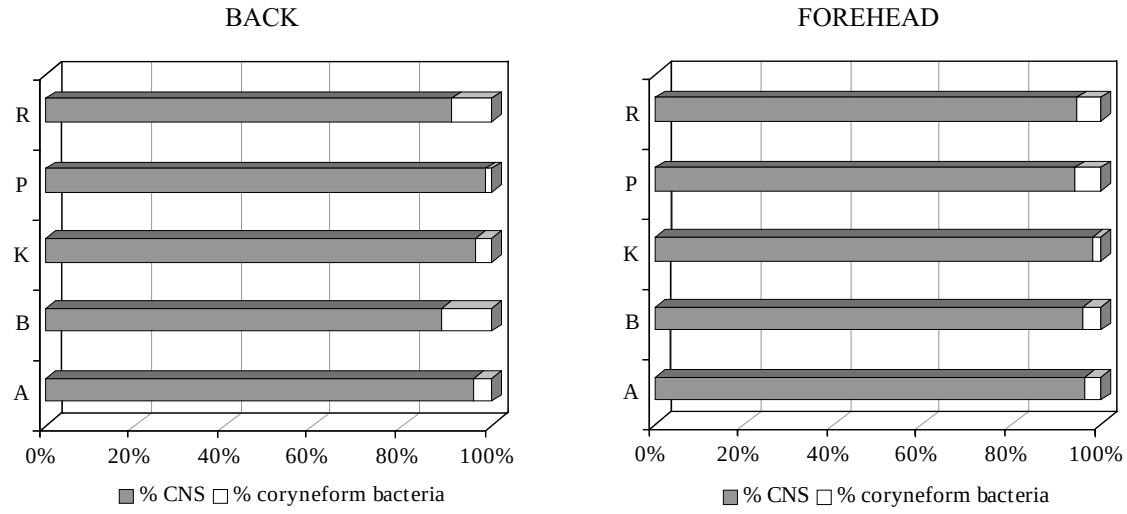

Fig.1. Percentage of coagulase-negative staphylococci (CNS) and coryneform bacteria on the back and the forehead

Closer analysis of coryneform bacteria showed that lipophilic and nonlipophilic coryneform rods and genus *Corynebacterium* all have the constant share no matter of its source (Table II). So it can be assumed that their population on the human skin is quite constant as to both quantity and quality if compare with the quantity of CNS. The ratio was constant and independent of the habitat of bacteria (the forehead or the back). Further analysis was conducted to introduce new data concerning the diversity of isolates from coryneform group on the human skin and to estimate which of them have the longest life period. The investigation was being conducted for 5 months; during this period 6 samples were taken from each person.

Due to the lack of adjusted taxonomy of coryneform bacteria and consequently the lack of simple methods of differentiation of such diverted population the new schema of biotyping based on the 9 tests was introduced (Table III). The schema was found for the most often described features that allow to classify

Table II
Coryneform rods on the back and the forehead

Groups	Forehead	Back
Total	278	196
Lipophilic isolates (%)	86.3%	84.7%
Nonlipophilic isolates (%)	13.7%	15.3%
Corynebacterium (%)	92.4%	93.4%

Table III
Tests used for identification and system of numeration of biotypes

Tests		Ascribed number		Example	
		Positive result	Negative result	Result	Number
Lipophilism		L	N	+	L
Nitrate reduction		1	0	–	0
Urea hydrolysis		2	0	–	0
Acid production	Glucose	4	0	+	4
	Maltose	1	0	+	1
	Sucrose	2	0	+	2
	Lactose	4	0	–	0
	Mannitol	1	0	–	0
Esculin hydrolysis		2	0	–	0
CAMP test		4	0	+	4
Code of biotype					L 4 3 4

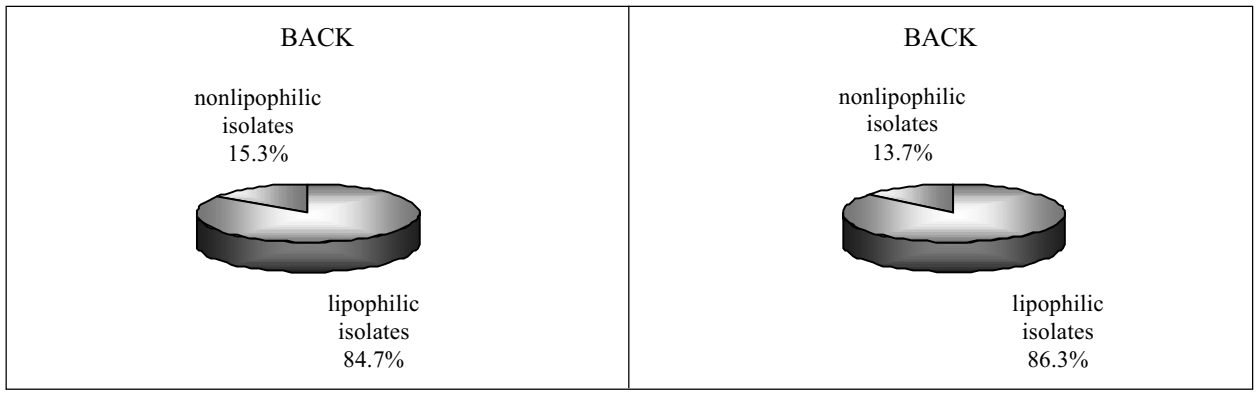

Fig. 2. Lipophilic and nonlipophilic coryneform bacteria on the back and the forehead

isolates with the high probability to the already recognised species of coryneforms connected with humans. It enabled giving the particular codes to the isolates and facilitated their classification.

Coryneform isolates were analyzed in two groups: lipophilic rods (in the code system they were described as L) amounted 85.7% and nonlipophilic (N) amounted 14.3% of all isolates from the examined specimens (Fig. 2).

In the group of lipophilic rods 22 biotypes were distinguished. Biotypical diversity of lipophilic isolates from coryneform group and their taxonomy is shown in the Table IV. On the basis of description provided by the literature on the subject 16 of the biotypes were classified to 5 species: *C. afermentans* spp. *lipophilum*, *C. urealyticum*, *C. kroppenstedtii*, *C. jeikeium*, *C. diphtheriae* var. intermedius and to three CDC taxa: CDC group G1, CDC group G2, CDC group F1. Isolates described by different codes may have been classified to the same group or the same species. Six biotypes of isolates described by different codes didn't find any representation in recently described species or groups.

Analysis of frequency of certain biotypes or groups in all six samplings from each patient is shown in the Table V. The most often isolated biotypes were L410 and L414 (*C. jeikeium*) and L430 and L434 (CDC group G2), followed by L 530 (group CDC G1) and L000 (*C. afermentans* spp. *lipophilum*) (Fig. 3). Isolates belonging to the one or both of biotypes differing from each other only with the result of CAMP test were found many times on the skin of all the individuals. These isolates are common, dominating in this group flora.

Isolates forming certain biotypes were diverted. Many of differences were connected with the cell morphology. Some of these differences, like the amount of metachromatic granules, size of the rods, snapping divisions, were observed in the microscopical image. Some differences were concerned with the characteristic of the colonies – their colour and type of the surface and also the image of synergistic haemolysis (CAMP). However, all these features were in accordance with the description of the certain species. It was assumed that isolates that differ phenotypically and also these which were from different samplings form

Table IV
Species/taxa of isolated lipophilic coryneform bacteria and their codes of biochemical biotypes

Species/taxa	Biotypes
C. afermentans spp. *lipophilum*	L000, L004
C. urealyticum	L200
C. kroppenstedtii	L400, L404, L424
C. jeikeium	L410, L400, L414
CDC group G2	L430, L434
CDC group G1	L530, L534
C. diphtheriae var. intermedius	L510
CDC group F1	L730, L630
Non identified isolates	L100, L210, L010, L024, L514, L124

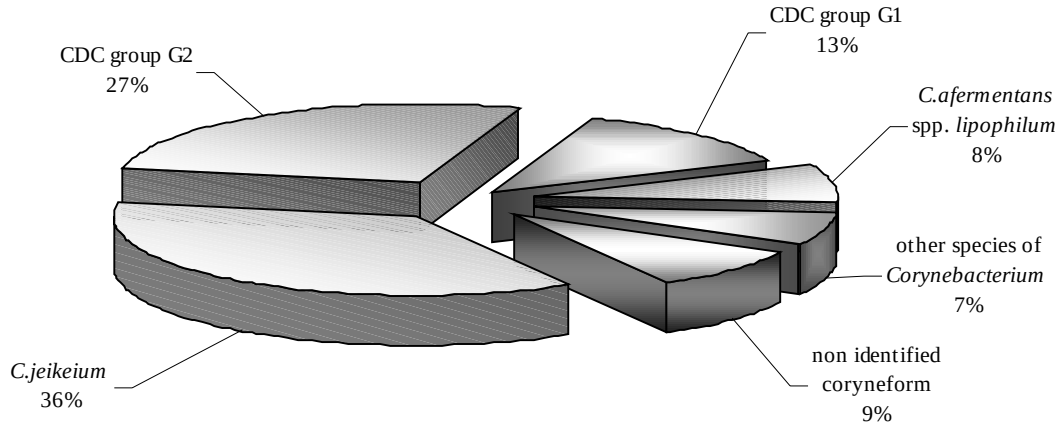

Fig. 3. Percentage of species/taxa of lipophilic coryneform bacteria

variations – separate strains. The frequency of isolation of certain biotypes, number of phenotypical variations and the number of all the isolates classified as particular biotype were shown in the Table VI. Among the largest biotype L410 (103 isolates) 49 phenotypical variations were found. According to the avalaible identification scheme all of these variations were classified as *C. jeikeium*. Biotype L414, very close to L410 differing only with the positive result of CAMP test, was not so numerous (44 isolates), but still it was represented in 19 from 30 samples; there were 31 of its phenotypical variations. Such biotypes like L430 and L434 differing from each other only with the result of CAMP test were also frequently isolated. Each of them was reperesented in 19 samples. Relatively to the number of isolates they had many phenotypical variations (see Table VI).

Nonlipophilic flora contributed to small part of the population of coryneform rods on the skin. In successive samplings rods of different species were isolated. None of them was observed to be dominating. There were 68 nonlipophilic isolates. Among them 27 biotypes and 17 species were separated. Species and adequate biotypes are shown in the Table VII. There were 17 isolates in this group that could not been identified on the basis of considered features. All the nonlipophilic isolates appeared in the single samples in very small amounts. This let to assume that they form separate strains.

Table V
The frequency of isolation of lipophilic biotypes from the skin of observed individuals

Species/taxa		*C. afermentans* spp. *lipophilum*	*C. afermentans* spp. *lipophilum*	*C. urealyticum*	*C. jeikeium* or *C. kroppenstedtii*	*C. kroppenstedtii*	*C. kroppenstedtii*	*C. jeikeium*	*C. jeikeium*	CDC group G2	CDC group G2	CDC group G1	CDC group G1	*C. diphtheriae* var. intermedius	CDC group F1	CDC group F1	Non identified	Non identified	Non identified	Non identified	Non identified	Non identified
biotype		L000	L004	L200	L400	L404	L424	L410	L414	L430	L434	L530	L534	L510	L730	L630	L010	L024	L100	L124	L210	L514
Person	A	4	1		1	1	2		6	5	5		4	1			2		1			
	B	2	1	2				5	2	6	3	3	1				2	1		1		1
	K	1	1					1	2	2	2	4	1	1			3					
	P	3	1	3		1		5	6	3	5	2	1	1	1	2					1	3
	R							3	3	3	4	5				2						1

Table VI
The frequency of isolation and the number of phenotypical variations among particular biotypes

Biotype	Species/taxa	Number of specimens	Number of phenotypical variations	Number of isolates	Percentage of all isolated coryneforms
L000	*C. afermentans* spp. *lipophilum*	10	15	23	4.9
L004	*C. afermentans* spp. *lipophilum*	4	7	10	2.1
L200	*C. urealyticum*	5	6	7	1.5
L400	*C. jeikeium* or *C.kroppenstedtii*	1	1	1	0.2
L404	*C. kroppenstedtii*	2	2	4	0.8
L410	*C. jeikeium*	14	49	103	21.7
L414	*C. jeikeium*	19	31	44	9.3
L430	CDC group G2	19	25	41	8.6
L434	CDC group G2	19	42	70	14.8
L424	*C. kroppenstedtii*	2	2	3	0.6
L530	CDC group G1	14	18	29	6.1
L534	CDC group G1	7	12	23	4.9
L510	*C. diphtheriae* var. intermedius	3	3	7	1.5
L730	CDC group F1	1	1	2	0.4
L630	CDC group F1	4	3	4	0.8
L010	Non identified	7	10	18	3.7
L024	Non identified	1	2	4	0.8
L100	Non identified	1	1	1	0.2
L124	Non identified	1	1	1	0.2
L210	Non identified	1	1	2	0.4
L514	Non identified	5	6	9	1.9

Table VII
The frequency of isolation of nonlipophilic species

Species	Biotypes	Number of isolates	Number of specimens
Actinomyces viscosus	N634, N430, N570, N534, N572, N530	6	6
Brevibacterium casei	N000	7	5
Corynebacterium amycolatum	N430, N400, N410, N530	5	4
Corynebacterium seminale	N634, N525, N424	5	4
Corynebacterium minutissimum	N430, N431, N410	4	3
Corynebacterium coyleae	N004	4	3
Corynebacterium falsenii	N004, N204, N600	3	3
Corynebacterium imitans or *C. seminale*	N534, N434	6	2
Corynebacterium afermentans ssp. *afermentans*	N000, N004	2	2
Corynebacterium striatum or *C.seminale*	N524	1	1
Corynebacterium thomssenii	N204	1	1
Corynebacterium diphtheriae var. gravis	N410	1	1
Dermabacter hominis	N470	1	1
Turicella otitidis	N004	1	1
Actinomyces neuii ssp. *anitratus*	N475	1	1
Corynebacterium pseudodiphtheriticum	N300	1	1
Corynebacterium xerosis	N530	1	1
Non identified isolates	N002, N664, N473, N423, N411, N450, N452	17	8

Discussion

The main assumption and aim of the present work was to analyse part of the bacterial flora which lives in the deeper layers of epidermis and to amount its constant part – so called residents. These are the bacteria that may penetrate organism during long-term hospitalization connected with breaking covers of the body due to the ingeration of foreign bodies (*e.g.* medical devices like shunts, catheters). In case of immunocompromised patients it can be the reason of the heavy opportunistic infection (Coyle and Lipsky, 1990; Funke *et al.*, 1997; Domínguez-Gil *et al.*, 1999; Knox and Holmes, 2002). It is well known that skin flora includes among others coagulase-negative staphylococci. There is no need to convince anybody of their significant role in opportunistic infections. Research presented in this publication shows that CNS and coryneform rods establish a kind of dynamic equilibrium on the surface of healthy skin. Literature gives us cases in which representants of both groups were isolated from the opportunistic infections. In 1994 Bayston *et al*. isolated from the hydrocephalus shunt both CNS and coryneform rods. They observed that these rods had, just like CNS, the ability to create biofilms *in vitro.* It can be expected that creation of mixed biofilm can be a significant way of colonization human organism by these two groups of bacteria. Authors pointed out multiresistance of the strains they had isolated (Bayston, Compton and Richards, 1994; Funke *et al.*, 1997; Knox and Holmes, 2002; Camello *et al.*, 2003).

After the analysis of the composition of isolates we did not notice significant differences in the composition of coryneform bacteria population isolated from different individuals (although there had been such differences as to the whole residential flora (Kaźmierczak and Szewczyk, 2004)). All the individuals had very similar composition of coryneform bacteria population, moreover there were no differences between the back skin and the forehead skin. It proves a great conservatism of this part of skin flora. Literature shows that very important in nosocomial infections are strains (species) belonging to lipophilic coryneforms: *C. jeikeium*, CDC group G and *C. urealyticum* (Coyle and Lipsky, 1990; Koneman *et al.*, 1997; Funke *et al.*, 1997; Knox and Holmes, 2002). Our research shows unequivocally that they build the core of corynebacteria of residental physiological skin flora.

However, the main problem concerning coryneforms is the lack of proper, contemporary taxonomy of coryneform bacteria. During a long period of time the only well known spacies of coryneforms was *C. diphteriae*. Still there is no full date about basic features of coryneform bacteria. Due to the too small number of well characterized strains there are no good diagnostic scheme and taxonomy of microorganisms belonging to the coryneform group is still labile. The condition of making description of new species is not only its genetical dissimilarity to other known species but also very thorough description of its phenotypical features. Such description should be based on the isolations of these strains in different laboratories, on different continents. Exchanging of scientific information should let gather the collections of strains suitable for fixing phylogenetic relation and confirming existence of the distinct species by genetic methods.

As a result, there are no fine diagnostic scheme. Propositions of some authors are fragmentary and often contradictory to each other (Coyle *et al.*, 1993; Martinez-Martinez *et al.*, 1995; Funke *et al.*, 1996; Renaud *et al.*, 1996; Rassoulian Barrett *et al.*, 2001; Bernard *et al.*, 2002). Many authors indicate that avalaible rapid tests (API coryne) are not very useful due to obsolete database which doesn't include newly appeared species (von Graevenitz *et al.*, 1994; Funke *et al.*, 1997; Funke *et al.*, 1997a; Renaud *et al.*, 1998; Rassoulian Barrett *et al.*, 2001). There is also very little knowledge about mutability among species, which is very significant according to the results present in this study. It is highly probable that identification in this group would be based on specialized diagnostic technics using methods of molecular biology (ex.16S rRNA probe) (Renaud *et al.*, 1996; Koneman *et al.*, 1997; Funke *et al.*, 1997; Tang Y-W *et al.*, 2000; Shukla *et al.*, 2003). However some authors that use these technics, like Renaud *et al*. (1998), indicate a strong need of differentiating based on phenotypical features. These authors proposed differentiation on the basis of assimilation of different sources of carbon. There is no doubt that there is a strong need for very basic researches that would allow to create proper classification scheme useful for routine laboratories and estimate danger from certain taxons.

The most serious publications dealing with description and classification of coryneforms have been issued in the USA and there where the first descriptions leading to separation so called CDC groups from which single, well described species evolved (Coyle and Lipsky, 1990; Koneman *et al.*, 1997). Yet casuistic data from the whole world show single strains which are the cause of opportunistic infections (Sjödén *et al.*, 1998; Funke *et al.*, 1998; Funke *et al.*, 1998a; Wattiau, Janssens and Wauters, 2000; Renaud *et al.,* 2001; Yassin, Steiner and Ludwig, 2002; Bernard *et al.*, 2002; Shukla *et al.*, 2003).

There has been said a lot about the urge of selection of additional tests differentiating species. One of such tests is a rarely used test of synergistic haemolysis CAMP. There is lack of literature dealing with

CAMP applied to newly reported species, *e.g. C. kroppenstedtii* (Bernard *et al.*, 2002). No reports about CAMP-positive strains of *C. jeikeium* and strains of coryneforms belonging to the CDC group G2 were found. Due to the data presented in this study CAMP-positive strains can appear among all of these species. However, the observed images of CAMP differed as to intensity and haemolysis markings. This phenomenon has been already described by the other authors (Koneman *et al.*, 1997).

Results presented here show the domination of *C. jeikeium* and rods from CDC group G2 among the coryneform rods on the skin of healthy human individuals. Although *C. diphtheriae* is the most notable species of *Corynebacterium*, it is *C. jeikeium* (formerly CDC group JK) which has become the most often isolated, clinically very important species of this genus. *C. jeikeium* has been also found in inanimate hospital environment (Coyle and Lipsky, 1990; Funke *et al.*, 1997). Infections caused by this microorganism were reported not only in immunocomprised patients but also as a nosocomial septicemia at immunocompetent patients. *C. jeikeium* is considered to be resistant to most of the antibiotics (Riegel *et al.*, 1994; Koneman *et al.*, 1997; Funke *et al.*, 1997). The antibiotic-resistance of this species is believed to be connected rather with chromosome than with plasmids (Funke *et al.*, 1997). Antibiotic-resistance is indicated by many authors as a factor important to identification of coryneform group (Bayston *et al.*, 1994; Brandenburg *et al.*, 1996). It should be, however, pointed out that there is hardly any data about natural resistance of these bacteria and the ways they acquired the resistance to the antibiotics.

Another most often represented taxa in our researched material was CDC group G2. Due to frequent isolation of CDC group G2 and CDC group G1 from clinical materials they are the subject of researches leading to evolve new species. Riegel *et al*. (1995), while applying DNA-DNA hybridization between the strains from CDC groups G1 and G2, noticed a great genetical similarity between them. These groups of coryneform are responsible for fatal endocarditis (Funke *et al.*, 1997). Williams, Selepak and Gill (1993) indicating that they are the second after *C. jeikeium* most common coryneform isolated from clinically significant cultures.

The coincidence between clinical significance of these two corynebacteria groups and their domination on the skin of healthy men tested is striking. It shows that the direction of our researches is right. Large number of variations among each of appointed biotypes demonstrates the big variety of received isolates. It shows that majority of them were not clones present in one sample, the descendant of the same cell living in the common ecological niche. On the other hand a large group of isolates that could not been identified according to the taken identification schema confirms thesis about great variation of coryneform rods being residents of skin flora and about the lack of information on this group of bacteria.

Completing the collection of well phenotypically characterized isolates will allow to undertake comparative studies leading to the description of new species, to find the conditions for isolations and construct simple identification scheme to recognise the most common strains causing nosocomial infections.

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