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Non-cultural methods of human microflora evaluation for the benefit of crew medical control in confined habitat

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ABSTRACT

Current control of human microflora is a great problem not only for the space medicine but also for practical health care. Due to many reasons its realization by classical bacteriological method is difficult in practical application or cannot be done.

To evaluate non-cultural methods of microbial control of crews in a confined habitat we evaluated two different methods.

The first method is based on digital treatment of microbial visual images, appearing after gram staining of microbial material from natural sample. This way the rate between gram-positive and gram-negative microbe could be gained as well as differentiation of rods and cocci could be attained, which is necessary for primary evaluation of human microbial cenosis in remote confined habitats.

The other non-culture method of human microflora evaluation is gas chromatomass spectrometry (gcms) analysis of swabs gathered from different body sites. Gc-ms testing of swabs allows one to validate quantitative and special microflora based on specific lipid markers analysis.

1. Introduction

Long space flights inevitably entail risks of various natures. The most significant factors for preservation of life, health and normal working capacity of crew members includes curbing the risk of infectious diseases. The concept of periodic accumulation of potential pathogenicity by microorganisms in the long time action of space flight factors, formulated earlier, solves this problem to a significant extent.

Diagnostics of the microflora condition-generated commensal microorganisms is one of the most rational ways of preventing the infection of cosmonauts onboard a space vehicle. In standard practice the classical bacteriological method is applied to receive similar information on a condition of a barrier colonization resistance, based on cultivation of bacteria allocated from integumentary tissues and their subsequent definition to species. However in conditions of space flight its use is impossible. The decision of microflora condition control problem demands creation of systems and the methods excluding necessity of bacteria cultivation.

The computer analysis of microbial cells images is based on automated analysis. It allows obtaining information on critical parameters (morphological, tinctorial, quantitative) and on the definite quantitative ratio of microorganism groups in the investigated material. The given method assumes analyzing open biotopes of human organism as the covering tissues (skin and mucous) are in the closest direct contact to the environment.

The normal ratio of researched microorganisms groups is known for every biotope. So knowledge of qualitative and quantitative structure of their microflora can give a complete representation about the current condition of immunity. This, in turn, allows predicting the risk of development of an infectious disease. The developed

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method yields live data about human microflora without separating and cultivating microorganisms on media, which reduces the test time from 2–5 days to 1.5–3 h. It permits one to give timely recommendation on correction of the immune status.

GC-MS permits one to obtain unique information regarding the composition of specific monomeric chemical compounds of a microbial cell. These markers can be detected in the mass of other chemical compounds in total biomass constituencies of biological objects and can be used for determining microorganisms in corresponding genera or species. The root cause of the problem lies in the direct extraction of Fatty Acids (FAs) from examined specimens by a chemical procedure by dividing them in the capillary column of gas chromatography and analyzing the compounds in a dynamic rate on mass-spectrometry. This procedure takes only 30 min as the chromatograph is combined with mass-spectrometry and provided by PC with corresponding programs of automatic analysis and taking into account the time of sample preparation and quantification of data (not more than 3 h). Result involves quantification determining of microorganisms' constituencies.

Today the FAs of the most significant microorganisms have been well investigated regarding its repeatability and its special and genera specificity have been estimated [1,2].

Diagnostic possibility of the method to detect microbial markers in clinical materials seems promising.

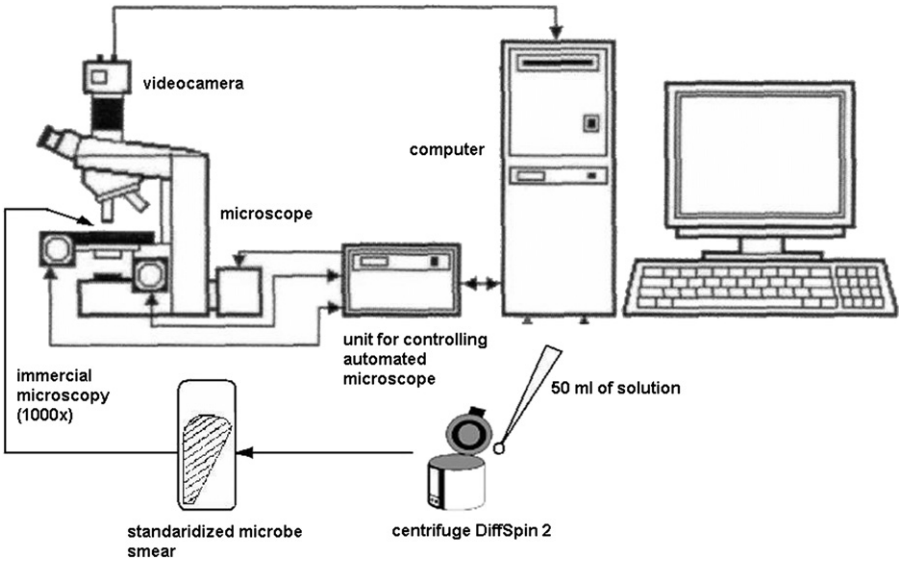

Fig. 1. The system for automated microbial analysis of native swab.

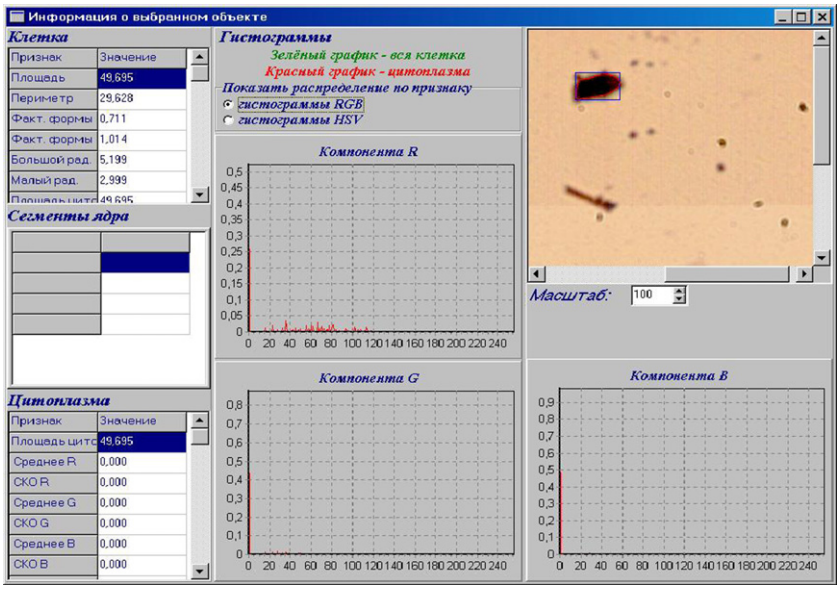

Fig. 2. An example of objects segmentation on vision field of native smear image.

Introduction of GC-MS permits one to reduce time but increase value of investigation, passing the stages of recurring replanting of primary colonies and test fermentations, which are particularly difficult, laborious and prolonged processes for anaerobic microorganisms. This method permits not only detecting marker compounds (FAs, aldehydes, alcohols and sterines) in pure cultures of microorganisms, isolated from clinic material, but also determining and calculating the composition of microbial community, which is encoded in markers of the concrete sample [5,6]. The material for investigation in clinic is blood, saliva, urine, liquor, synovial or ascetic liquid, mucosa, phlegm, throat, nose and ear swabs, secretion from genitals, tissue sampling, skin smears, depending on the aim of investigation. The skin biotopes could be selected in accordance with the concrete experimental task [4].

Samples of biological liquid or tissue should be treated immediately or frozen and stored at $-5/-18\,^{\circ}C$ in case it is impossible to immediately analyze. Transportation of samples at normal temperature for five hours can be permitted. Storing in dried form is needed in case the samples need distant transportation or need to be sent by post (dry at 70–85 °C).

2. Materials and methods

2.1. The automated analysis of native swab

By means of automated analysis method the laboratory researched 10 samples prepared from each of the 12 biotopes of five volunteers-participants of long-term experiment in confined habitat. Swabs from mucous membranes of the top respiratory ways were investigated.

The object of research for the automated analysis is smears prepared by means of centrifugation using a DiffSpin 2 Slide Spinner (model M 701-22, StatSpin, USA). The analysis was carried out by automatic scanning of the preparation under the microscope equipped with the automated subject table and a video camera. The system automatically focuses by the microscope, inputs the images of the microscopic vision field in computer memory, segments the image (allocation of objects from a background) and recognises it as gram-positive and gram-negative cocci and rods. Further the system counts quantity of microbial objects in groups (types) on all areas of scanning and calculates the number ratio between groups and the common microbial number (CFU/sample).

For verification of the data received by means of the automated analysis, bacteriological research was carried out. The material from biotope was passed through a rinsing solution and further crop from the received cultivations on nutrient mediums a blood agar of 5%, a mannitol hydrochloric agar, Endo and Sauburaud was spent. Crops incubated in thermostat at 37 °C for 2 days, then calculation of the evolved colonies and their specific definition was spent.

Also smears were investigated by means of immersion microscopy ($1000\times$). The common condition of swab was estimated on the following scale: "low", "average", and "high" contamination level. "Low" semination corresponded to 1–2 microbial objects on all smear areas,

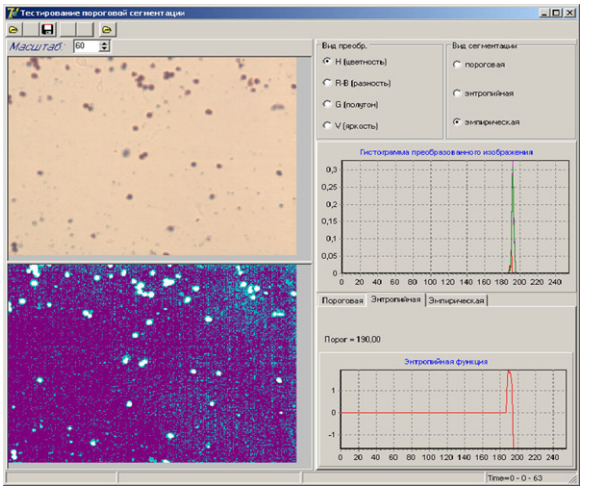

Fig. 3. The vision field of Gram-positive cocci smear image processing.

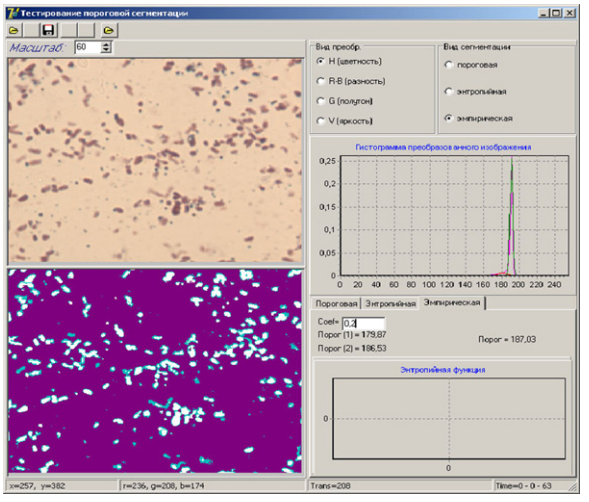

Fig. 4. The vision field of Gram-positive rod bacteria smear image processing.

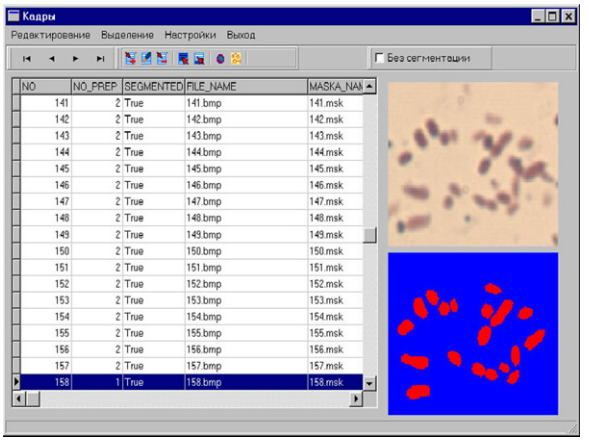

Fig. 5. The vision field of yeastlike mycoses smear image processing.

"average" – in fields of vision of a microscope on a regular basis there are microbial objects, "high" – microbial objects meet almost each field of vision of a microscope.

The scheme of the investigation equipment is shown in Fig. 1. An example of objects segmentation on vision field of native smear is given in Fig. 2. Examples of vision field with different types of microbial objects processing are given in Figs. 3–5.

2.2. Gas chromatomass spectrometry

We investigated microflora of cosmonauts, participants of one of the ISS mission one day prior to launch and on the 7th day of the flight. Material was taken with the use of standard packs including 12 biotopes and 1 control.

We investigated nose, mouth, tongue, teeth-mucosa of organism, ear, forehead, cheek, neck, chest, armpit, groin, and arm-skin of organism. Material was taken before flight and on the 7th day of flight.

Tampons with smears were divided into two subsamples, the first of which was directed to GC-MS investigation and the second was analyzed by the classic bacteriological method.

Samples preparation.

Samples in Teflon-lined glass test tubes were heated in 400 μl of 2 M methanolic HCL at 80 °C for one hour.

Subsequently 400 μl of hexane was added to the reaction mixture. The mixture was vortexed and stored until division of phases for 5 min at room temperature. The upper hexane fraction was evaporated for 5–7 min at 80 °C.

The dried residuum was treated with 20 μl of N, O-bysftoracetamide for 15 min at 80 °C in a closed vial.

80 μl hexane was added to the reaction mixture and the mixture was transferred to the closed vial. The mixture of esters of 2 mkl was injected into the capillary column of system AT-5973N Agilent Technologies by an autosampler. For treatment of the results the standard programs of the unit were used. GC-MS extracting of sample was on a capillary column with methylsilicon phase HP-5MS Agilent. The regimen of analyzing and detecting microorganisms by markers was implemented as described above. The analysis protocol and compatibility of markers to known microbial specia were performed in accordance with the description in [3,4].

Areas of markers "peaks" were integrated automatically by special program with an internal standard. Subsequently these data were involved in the quantification program, made in electron tables EXEL. Dates of calibration on dateric tridecan acide and pure cultures of clinical isolated microorganisms were used for quantification analyses.

2.2.1. Calculation of composition and amount of effective cells of microorganisms.

This mode is described for estimating ecological microbial communities in general view from m compounds and n specimens of microorganisms by composing system of m equation for n unknowns.

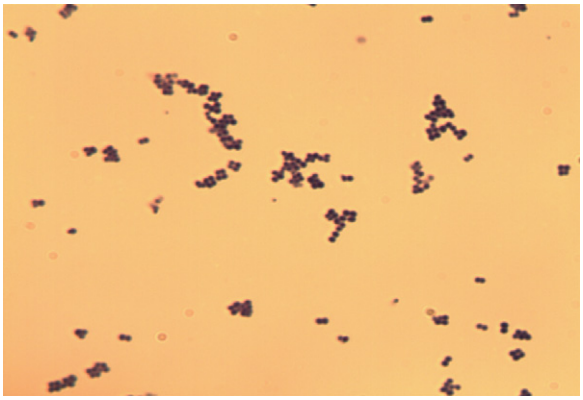

Fig. 6. The image of Gram-positive cocci.

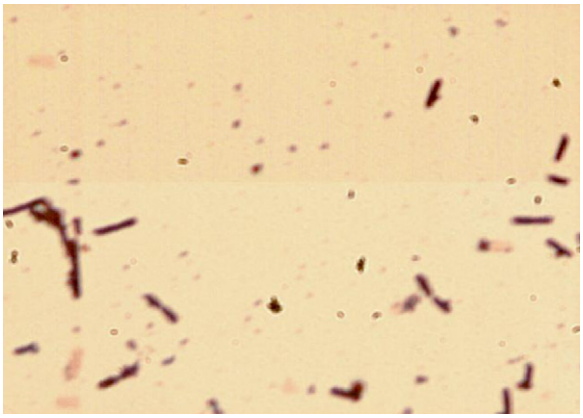

Fig. 7. The image of Gram-positive rod bacteria.

Table 1
Comparative results of human microflora testings using automated analysis of native swab and cultural method.

Specimen	Biotope	Automatic analysis	Expert appraisal	Cultural investigation
1	pharynx	$<10^4$	Low	$2{,}64\times10^4$
2	pharynx	$3{,}55\times10^6$	High	$2{,}02\times10^6$
3	pharynx	$5{,}45\times10^5$	High	$1{,}84\times10^5$
4	pharynx	$<10^4$	Low	$1{,}20\times10^3$
5	pharynx	$1{,}15\times10^5$	Medium	$4{,}56\times10^4$
6	nose	$<10^4$	Low	$2{,}40\times10^2$
7	nose	$7{,}8\times10^4$	Medium	$1{,}24\times10^5$
8	nose	$<10^4$	Low	$7{,}20\times10^3$
9	nose	$8{,}90\times10^4$	Medium	$6{,}60\times10^4$
10	nose	$1{,}10\times10^6$	High	$8{,}19\times10^5$

Table 2
Composition of microflora before and after flight. GC-MS analyses.

микроорганизм	Tongue Before	Tongue After	Ear Before	Ear After	Forehead Before	Forehead After	Mouth Before	Mouth After	Teeth Before	Teeth After	Nose Before	Nose After	Cheec Before	Cheek After
Streptococcus	0	0	359	0	0	382	0	0	575	0	710	0	787	0
Eubacterium lentum (группа А)	231	473	0	3905	532	0	62	872	34	302	2000	0	0	0
Bacillus cereus	104	1755	2024	190	2519	5997	122	370	129	843	206	1954	*4062*	*3486*
Pseudomonas aeruginosa	43	745	16	0	0	103	71	1098	71	722	78	149	9	55
Clostridium propionicum	963	6859	15820	14127	18217	37850	729	9372	609	3093	1477	18574	21460	14635
Stenotrophomonas maltophilia	0	0	0	98	46	22	0	72	0	73	0	34	0	16
Bacteroides hypermegas	83	278	2	43	0	21	14	199	76	255	0	79	0	22
Актиннмицеты	165	452	3019	2967	3700	5424	148	738	86	280	208	2890	3580	2876
Pseudonocardia	192	418	3020	2860	3079	4122	148	657	122	351	167	3054	2700	2350
Streptomyces	*578*	*403*	*0*	*0*	*0*	*0*	*686*	*0*	*847*	*372*	*184*	*0*	*0*	*0*
Clostridium ramosum	3006	7380	8029	0	0	0	2196	12818	2952	7458	**0**	0	0	0
Fusobacterium/ Haemophylus	17	3235	31	430	0	210	134	1906	74	2488	143	751	39	222
Alcaligenes	285	1852	145	2399	0	349	173	4460	131	3015	322	602	154	349
Rhodococcus	625	721	6376	7706	8447	8297	447	2077	281	865	550	8284	7663	5971
Staphylococcus intermedius	799	0	0	955	0	0	1030	234	1008	110	683	0	0	0
Corineform CDC-group XX	311	14	625	1024	655	37	275	327	382	93	235	744	236	271
Lactobacillus	1900	0	0	1257	452	658	1218	0	888	0	1212	1034	918	881
Campylobacter mucosalis	78	0	669	587	947	1351	104	94	51	61	48	1513	945	519
Mycobacterium/Candida	819	308	4227	4472	4630	6485	378	768	560	313	613	4903	6141	4127
Cl.difficile	433	480	435	549	226	207	536	436	502	508	866	372	287	351
Prevotella	690	5595	75	987	0	526	680	3014	615	2497	536	1048	35	804
Staphylococcus	224	189	807	591	851	563	216	276	173	307	205	726	1139	638
Helicobacter pylori, h18	548	2495	199	396	89	405	649	1769	534	2373	1025	902	178	527
Clostridium perfringens	161	868	61	167	37	237	182	703	150	617	428	508	75	203
Enterococcus	1176	4593	700	0	261	103	1671	0	1685	0	552	269	257	1897
Eubacterium	9	0	2	59	25	25	7	240	9	70	34	0	20	35
Propionibacterium spp	415	5204	298	0	0	686	451	3542	510	3292	542	1675	500	1467
Streptococcus mutans	495	527	455	1212	667	532	998	0	196	455	222	269	787	255
Nocardia asteroides	0	0	0	391	430	682	0	0	0	0	0	389	0	194
Propionibacterium acnes	96	0	26	0	0	4737	162	0	95	0	71	345	493	1814
Ruminicoccus	1821	0	1119	1567	3172	1914	0	2462	239	0	8355	0	1222	789
Actinomycetes 10Me14	680	1530	7282	4771	9403	13631	616	2601	444	1469	733	9890	9654	6587
Enterococcus	96	0	26	0	0	4737	162	0	95	0	71	345	0	0
Aspergillus	0	0	42	0	0	179	330	0	284	0	0	971	27	823
Total	49779	18899	55338	62525	66359	111910	15491	53373	15902	34541	23638	67991	72298	

The area of GC-MS marker peak is in proportion to its concentration. Therefore it is in proportion to concentrations of corresponding microorganisms, which is defined as the member of N cells in the unit of volume or weight of sample by the following formula:

$$N_1 = Ai[M_{st}/(q2 \times M_{sam} \times A_{st})]/Ri_1,$$

where the form in square brackets is constant

$$\kappa = \mathrm{M_{st}/q2/M_{sam}/A_{st}} = \mathrm{M_{st}(mg)/5,1} \times 10(-15)\mathrm{g/M_{sam}(mg)/A_{st}}$$

In these formulae A_i is the area of markers peak, M_{st} the quantity of standard, injected into the sample (mg), M_{sam} the corresponding quantity of the sample, A_{st} the area of standard peak, R_{il} the portion(%) of marker with index i in FAs profile of the determined microbe number 1 (N1),

q_2 the coefficient that is equal to 5.1×10^{-15} g, where value 5.9×10^{12} cells of microbes contained in 1 g of microbial biomass and part of FAs in cell, averaging 3%.

Correspondingly, the number of cells of any further microorganism can be calculated by the analogous formula $N2 = A_i \times k/R_{i2}$, multiplying areas of marker peak A_i on the k coefficient and dividing on the portion of markers (in %) in composition FAs of this microorganism.

By this mode an effective number of bacteria species, namely Clostridium perfringens, Cl. difficile, Prevotella, Stenotrophomonas, Klebsiella, Eubacterium, Rhodococcus, Nocardia, Sphingobacterium, Pseudomonas, Enterococcus, Staphylococcus, Enterobacteriaceae, etc., were detected. Some bacteria interfere in the synthesis of stearines in human cells, and it allows us to determine bacteria Eubacterium by the product of transformation of human cholesterol by its fermentation in coprostanol.

Some compounds do not have the property of marker, i.e. can be concerned with two or more taxons; therefore in this case part of the contribution of each microorganism should be determined, using conclusion of equation' system for two compounds.

Investigation was done by the classic bacteriologic method (plating microscopy, estimation of results by quantitative method, estimation of results) according to the method described in order MZ № 535 22.04.1985. Also

Table 3
Microbial state of cosmonauts' microflora in 7 days of spaceflight, GC-MS analyze.

Biotope	Before flight			7th day of flight		
	Microbe	CFU/ml	Hemolysis	Microbe	CFU/ml	Hemolysis
Nose	*E. coli*	3,00E+02	–	*E. coli*	4,00E+06	–
	S. epidermidis	5,00E+01	–	*Klebsiella spp.*	2,00E+06	–
	S. aureus	2,00E+01	–			
	Neisseria spp.	3,00E+01	–			
Throat	*Proteus spp.*	1,00E+06	–	*Proteus spp.*	1,00E+07	–
	S. epidermidis	6,00E+01	–			
Dental plague	*E. coli*	2,00E+01	–	*Proteus spp.*	5,00E+07	–
	S. aureus	2,00E+01	–			
	Proteus spp.	1,00E+06	–			
	Enterococcus spp.	5,00E+01	–			
Tongue	*E. coli*	1,00E+04	–	*Proteus spp.*	1,00E+07	–
	Proteus spp.	1,00E+06	–			
	Enterococcus spp.	5,00E+02	–			
	S. aureus	2,00E+01	+			
	Neisseria spp.	5,00E+01	–			
Ear	*S. aureus*	3,00E+01	+	*S. aureus*	2,80E+03	–
	S. epidermidis	4,00E+01	–	*Klebsiella spp.*	2,90E+04	–
	Enterococcus spp.	2,80E+01	–			
	Neisseria spp.	8,00E+01	–			
Forehead	*S. epidermidis*	1,40E+01	+	*S. epidermidis*	4,80E+03	–
	Neisseria spp.	2,20E+01		*P. aeruginosa*	2,00E+03	+
Cheek	*S. epidermidis*	7,80E+01	+	*S .aureus*	1,70E+04	–
	Neisseria spp.	1,00E+02	–	*S .epidermidis*	3,20E+03	+
Neck	*S. aureus*	2,30E+01	+	*S. aureus*	3,20E+03	+
	S. epidermidis	3,50E+01	–	*S. epidermidis*	6,10E+03	–
	Aeromonas hydrophila	1,00E+01	–	*Entrrococcus spp.*	6,30E+03	–
	Neisseria spp.	1,00E+01	–	*Neisseria spp.*	8,00E+01	–
Chest	*S. aureus*	3,00E+01	+	*P. aeruginosa*	1,20E+02	+
	S. epidermidis	4,00E+01	–	*P. aeruginosa*	6,40E+03	–
	Neisseria spp.	6,50E+01	–			
Axilla	*S. aureus*	1,00E+04	+	*S. aureus*	9,20E+04	+
	E. coli	1,00E+00	–			
	Klebsiella spp.	2,00E+00	–			
Perineum	*S. aureus*	5,00E+03	+	*S. aureus*	2,00E+05	+
	E. coli	3,00E+00	–	*Neisseria spp.*	1,20E+05	–
	Enterococcus spp.	3,00E+01	–	Transparent Gram-	4,00E+06	–
Arm	*S. aureus*	1,20E+01	+	*S. aureus*	5,60E+02	–
	E. coli	2,00E+00	–			
	Enterococcus spp.	2,20E+01	–			

isolated cultures were identified (using corresponding methods and media) according to the order MZ №535 22.04.1985. Biochemical identification was carried out using commercial test systems LACHEMA, according to the instruction of the manufacturer.

3. Results and discussion

3.1. The automated analysis of native swab

The results received by means of the automated analysis were compared with received cultural method and with expert estimations. These researches have shown good correlation with previously mentioned methods. It was shown that results of the automated analysis are authentic at number 10^4 CFU/sample and above. Such accuracy is sufficient and allows revealing the threat of an infection in time. Results of the researches are presented in Table 1. The image of gram-positive cocci and rod bacteria are shown in Figs. 6 and 7.

3.2. Gas chromatomass spectrometry

The results obtained by GC-MS are presented in Tables 2 and 3.

The dates of GC-MS analyses in most cases compared with bacteriological indexes:

increasing of quantitative compounds of protective groups:

- Growth of Pseudomonas aeruginosa,
- Reduction of Enterococcus spp.

Certain deviations from bacteriological studies occur in quantitative characteristics of tested cultures. There was no distinguishing of S aureus from Staphylococcus spp. pool. Also there is a problem with distinguishing Protea

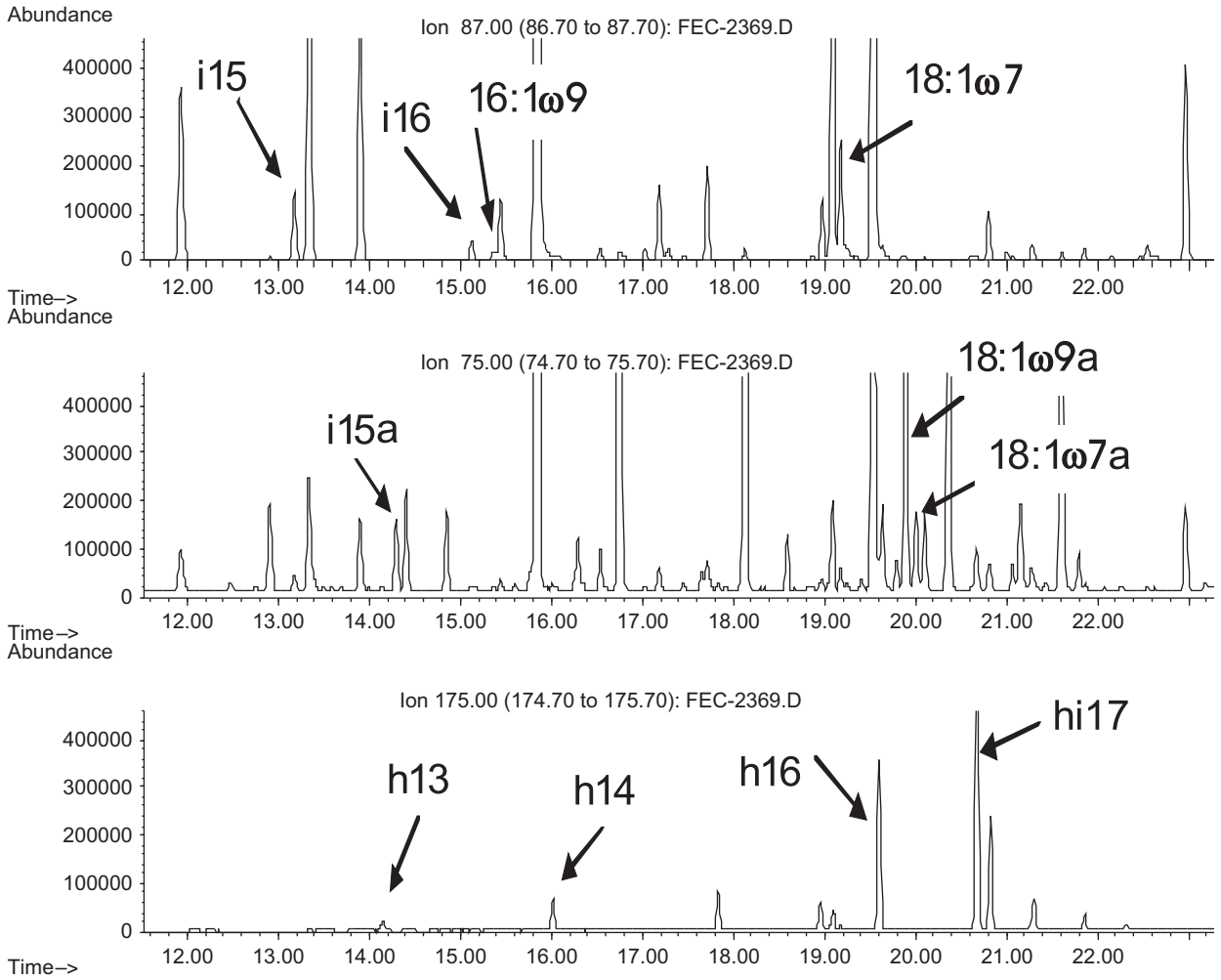

Fig. 8. Single ion monitoring chromatograms represent fractions of a cavity of nose fatty acids (ion 87, upper plot), fatty aldehydes (ion 75, in the middle) and hydroxyl FA (ion 175, lower plot). Marking: 18:1ω7a-18- is the number of carbon atoms, the figure after colon denotes the number of double bonds; ω7 – double bond position from carboxyl head of molecule h – 3-hydroxy-acid, back a – aldehyde, front i – *iso*-branched. For example, hi17 means 3 -hydroxy-*iso*-heptadecanoic acid.

from Providencia and detection of Klebsiella. Anyway it was the first attempt to use GC-MS method for covering tissues microflora detection and the obtained results were satisfactory in general.

The chromatograms obtained by the selective ion method (Fig. 8) can confidently detect microbial components in the presence of predominant human wastes components. For the most part, the peaks of target substances are superposition-free, rather distant from the peak of a substrate and therefore they may be accessible for automatic random integration in accordance with the standard program of the GC-MS system. The list of fatty acids, aldehydes and sterols detected in specimens is given elsewhere [7], by indicating the most likely microorganisms in whose cells they usually were found. For instance, i16 (*iso*-hexadecanoic acid) is a marker of *Streptomyces* spp., 18:1ω7 refer to Lactobacillus spp., h14 (3-hydroxy-tetradecanoic acid) is a well-known summed feature of family *Enterobacteriaceae,* genera *Haemophylus, Fusobacterium* and other gram-negative microbes, whose endotoxine contains this substance.

4. Conclusions

The proposed 2 non-cultural methods could be a good perspective for these purposes.

With the aid of chromatomass–spectrometry method testing it was confirmed that on the 7th day of flight there is an increase of pathogenicity potential, i.e. quantitative increase of conventional pathogens and decrease of protective groups. The method allows one to increase the spectrum of the controlled list of microbial specie. The data in majority are comparable with bacteriology indices: quantitative increase of Pseudomonas aeruginosa, decrease of enterococci. This determines the necessity of maintaining a portable chromatomass spectrometer for diagnostics of disbacteriosis.

Problems that should be solved concern the problems of differentiation of certain microbial groups: differentiation of S. aureus from the other staphylococci, detection of Proteus spp., Klebsiella spp. Quantitative characteristics of microbial content should be revised. Also one and the same markers are used for detection of different specie. As a result in some cases "false detection" occurs.

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