MICROBIOLOGICAL ASSESSMENT OF THE CLINICALLY SIGNIFICANT CULTURES OF THE OPPORTUNISTIC MICROORGANISMS ISOLATED FROM THE INTESTINES OF THE PATIENTS WITH ALZHEIMER'S DISEASE

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Annotation. *In the microbiological study of the intestinal microflora of the patients with Alzheimer's disease colonization resistance disorders of the mucous membrane of the intestines have been revealed: forming of the multicomponent associations, containing yeast fungi and opportunistic pathogenic bacteria (64.7% of all strains were characterized by the high level of their adhesive activity). Dysbiotic disorders of the intestinal microflora of the patients with Alzheimer's disease were followed by authentically low ($p < 0.05$) quantitative indices of the indigenous microbiota content: Lactobacillus spp. 4.48 ± 0.15lg CFU/g against the background of the III dysbiosis degree; Bifidumbacteium spp. 3.7 ± 0.2lg CFU/g p – II dysbiosis degree. In vitro tests of Lactobacillus spp. and Bifidumbacteium spp., isolated from the patients with Alzheimer's disease, have not revealed any strain with a high degree of adhesion and the high "++++"level of antagonistic activity, concerning opportunistic clinical strains of E.coli, P.vulgaris, K. pneumoniae., isolated in the clinically significant concentration.*

Key words: *Alzheimer's disease, intestinal microbiome, antagonism, adhesion.*

Introduction. In the medical science neurology and microbiology have substantially developed on accurate parallel trajectories, interacting only in pathological situations, when there was a direct infection of the central nervous system. However, in the last decade there was a revolution in biomedicine on understanding that the gut microbiota (trillions of bacteria, living in the intestines) plays a key role in the maintenance of homeostasis and in programming of the main systems of the organism, including human brain as well [1].

The growing volume of researches is concentrated on the coverage of the bidirectional ways of interrelation between colibacilli and the central nervous system, "microbiota-gut-brain" interrelation, however this area is in the initial stage of development [2]. Changes in microbiomes, its metabolites and the "gut-brain" interaction are connected with a wide range of diseases, including various disorders of brain functioning. Studying of a microbiome demands the tight cooperation of doctors with scientists in fundamental sciences and bioinformatics and is the most effective when traditional disciplinary barriers between neurology, gastroenterology and microbiology are destroyed [1].

The digestive tract represents an open circuit, which constantly interacts with

environment microorganisms, generally due to the receipt of food and water. The big area of the mucous barrier of the digestive tract becomes populated by microorganisms practically right after the birth of the person, and during the whole life microbic associations is the major microecological factor for health maintenance [3].

The main physiological functions of the normal microflora are: digestive, power and morphookinetic (epithelium power supply, thermal organism providing, regulation of the vermicular movement of the intestines, differentiation and regeneration of epithelial tissues of the mucous membrane). The process of digestion can conditionally be divided on actually, that one, carried out by the own enzymes of the organism, and symbiotic digestion, occurs with direct participation of the intestinal microflora. Intestinal microflora participates in fermentation of the food components, which were not split earlier, mainly carbohydrates – starch, oligo- and polysaccharides (including cellulose) and also proteins and fats [3].

A set of experimental works are directed to the attempts of the analysis of the ways of communication between the gut and the brain. Bacteria of the intestines influence the central processes through a variety of mechanisms. First, the ability of microbiota to synthesize neurotransmitters (that is, γ-aminobutyric acid, noradrenaline and dopamine) is the important communication medium. Secondly, microflora is of the main value in activation of the immune system, which can play the main role in aging, neurologic disorders and neurodegeneration. At last, microflora synthesizes metabolites, including short-chain fatty acids, which are necessary for intestines, the immune system and potentially – for the health of the brain [1].

Besides, the gut microbiota and brain are connected through the vagus nerve and through the regulation of the main food amino acids, such as tryptophane. Considering a close connection between the gut microbiota and brain, there is no wonder that colibacilli play a key role in neurologic and mental diseases [1].

Alzheimer's disease is the most widespread degenerative disease of the brain. It is supposed that Alzheimer's disease has its polietiological character. In case of the early onset of the illness the major etiological factor is genetic severity. Today three pathological genes, which posession provides 100% risk of getting Alzheimer's disease at the age till 60, are known. They are: the gene, coding protein – the predecessor of amyloid (the 21st chromosome), presenilin-1 (the 14th chromosome) and presenilin-2 (the 1st chromosome). The senile form of Alzheimer's disease (the beginning if after the age of 60) is partially connected with another pathological gene – anoF4 (the 19th chromosome) [4].

Except genetic predisposition, the risk factors of Alzheimer's disease is arterial hypertension, diabetes mellitus, abdominal obesity, the lack of B12 vitamin, depression and craniocereberal trauma in past history, sedentary life, the low level of education and intellectual activity [4].

The concept, according to which microflora can be of great value in Alzheimer's disease pathophysiology is not new, and the idea that amyloid, which congestions is one of key symptoms of Alzheimer's disease, can act as an antimicrobic peptide in the brain, is an fascinating concept [5]. However, according to Koch's postulate it is

ethically difficult to prove if there is an infectious reason of neuroinflammation and neurodegeneration. Like in case of Parkinson's disease, interrelation between intestinal proteins and brain health there is a special attention with understanding that the amyloid-like proteins can be developed by bacteria, that increases α-synuclein pathology in old rats and worms [5].

Studying of the biological properties of the opportunistic microorganisms and representatives of indigenous microflora, isolated from the intestines of the patients with Alzheimer's disease, became the objective of our research.

Materials and methods. During the research the condition of the intestinal microflora of the patients with Alzheimer's disease (n = 21) and elderly people – a reference group (n = 21) was determined according to methodical instructions [6]. The specific identification of bacteria was carried out according to Bergey's determinant [7]. Studying of the adhesive activity of clinically significant isolates of bacteria was carried out by V.I. Brilis technique and coauthors. [8]. The level of adhesiveness of the studied bacteria was estimated, proceeding from adhesion index of microorganisms (AIM). In particular, the microorganisms, which were non-adhesive had AIM <1.75, the low-adhesive – AIM from 1.76 to 2.5, medium-adhesive – from 2.51 to 4.0 and high-adhesive – when IAM ≥ 4.1 units.

For studying of the adhesive ability of Candida yeast fungi the model of the buccal epithelium with creation of the following conditions was chosen: the temperature of 37 °C, the contact epithelium of the oral cavity of healthy people, pH of the culture medium – Sabouraud dextrose agar, adhesion to the buccal epithelium for all strains was carried out at pH 7, 0.

The antagonistic activity of Lactobacillus spp. and Bifidumbacteium spp. isolates relatively to clinical isolates of opportunistic pathogenic bacteria (Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae) was carried out by the method of perpendicular strokes (delayed antagonism).

Statistical processing of the obtained results was carried out by means of the Statistica 6.1 software package with the use of parametrical Student's t-test.

Results and discussion. After obtaining results of the microbiological research of intestinal microflora all the patients with Alzheimer's disease were divided into 3 groups: 38.1 ± 0.04% of the examined patients had dysbacteriosis of the I degree (1 group); 28.6 ± 0.03% - the II degree (the 2nd group) and 33.3 ± 0.03% – the III degree (the 3rd group).

Dysbiotic shifts of the I degree, as a part of the intestinal microflora of the patients with Alzheimer's disease (the first group) in 75.0 ± 0.1% of cases had their latent, compensated character, which was characterized by minor quantitative changes in optional and anaerobic and the indigenous (bifido-, lactoflora) parts of the intestinal microflora and also an absence of the intestinal dysfunctions according to the anamnesis. In other patients of this group (25.0 ± 0.1%) in past history the intestinal dysfunction, which was shown in the form of diarrhea, was registered.

The patients of the 2nd group with the II degree of dysbiotic changes (28.6 ± 0.03%)

showed the subcompensated form of dysbiotic disorders, which was characterized by the quantitative and qualitative changes of Escherichia coli population (in comparison with the indices of the reference group), namely the quantitative increase in Escherichia coli with the low enzymatic activity (up to 7-10% of the total number of E. coli) and a reliable decrease ($p < 0.05$), in comparison with the indices of the reference group, Escherichia coli population degree of the intestines with the normal enzymatic activity to 5.7 15lg CFU/g.

33.3 ± 0.03% of the patients of the 3rd group (decompensation form of dysbiosis) showed a reliable decrease in the degree of obligate anaerobic bacteria population in the intestines – Bacteroides spp., Fusobacterium spp., Peptostreptococcus spp., ($p < 0.05$), in comparison with the indices of the reference group and these indices were 2 - 3.5 times lower than the indices of the reference group. It should be noted that in 71.4 ± 0.02% of such patients a reliable increase ($p < 0.05$) in the population degree of Clostridium spp., in the intestines, namely C. difficile up to 6 lg CFU/g. (the index of the reference group is lg 5 CFU/g.) was registered. Besides, in the patients of the 3rd group the intestinal microflora was characterized by a sharp qualitative and quantitative (< 0.05) reduction of Escherichia coli population degree with the normal enzymatic activity to 4.2 lg CFU/g (the index of the reference group 7-8 lg CFU/g) and the quantitative prevalence of opportunistic microorganisms: Klebsiella spp.; Proteus spp.; Cirtobacter spp.; Enterococcus spp., S. aureus, Morganella spp., Providencia spp., Hafnia spp., Candida spp. – the total population index was ≥ 9 lg CFU/g (the total exponent of the population of the intestines of people of the reference group was 4-4.5 lg CFU/g).

An important role in formation of biocenoses of a human body belongs to opportunistic microorganisms, which specify a considerable part of the normal microflora of the organism. Performing various functions, these microorganisms are quite often capable to cause various pathological processes not only in the biotopes, but also in other organs and systems. It is known that the representatives of the normal microflora, possessing certain mechanisms of adaptation, are capable to cause diseases against the background of the immunity decrease. In this regard they can be called opportunistic; approaches to studying of the factors of colonization have to be similar to those ones, when studying the corresponding factors in pathogenic bacteria [9].

The value of adhesive characteristics of the bacteria for a macroorganism can be considered from two positions. On the one hand, the adhesive potential of the indigenous microflora is one of the factors of realization of colonization resistance of the mucous membrane of the intestines and prevention of joining of pathogenic microorganisms to the receptors of the mucous membrane. On the other hand, in case of development of dysbiotic disorders the adhesive properties of the opportunistic microflora are considered as a pathogenicity factor, as they allow the microbes to be fastened up on the surface of the skin, mucous membrane and to colonize this biotope, reaching a certain population level [10-11].

The results of specifying the indices of Lactobacillus spp. (N = 41) and Bifidumbacterium spp. (N = 33) adhesion to erythrocytes of blood 0 (1) of the blood

type, isolated from the patients with a different degree of dysbiotic changes showed that there were no any strain with a high adhesion degree (Tab. 1).

Table 1

Distribution of the cultures of indigenous microflora, isolated from the intestines of the patients with Alzheimer's disease, according to their adhesive properties

Studied strains	Adhesion degree		
	low-adhesive	medium-adhesive	high-adhesive
	The number of strains (%)		
Lactobacillus spp. (n = 41)	65,9	34,1	0
Bifidumbacterium spp. (n = 33)	42,4	57,6	0

In the patients with Alzheimer's disease against the background of various disorders of colonization resistance of the mucous membrane of the intestines, which is mediated by the representatives of the indigenous microflora, formation of the multicomponent associations are observed. They consist of yeast fungi and opportunistic pathogenic bacteria and are evident in clinically-significant quantities (the population degree authentically ($p < 0, 05$) was higher in comparison with the same indices of the patients of the reference group. The results of determination of the adhesive properties of the studied strains of opportunistic microorganisms are shown in table 2.

The analysis of the results of the study on the quantitative level of opportunistic microflora has showed that the population degree of such microorganisms in the intestines was higher in the patients of the 3rd group in comparison with the 2nd one. So, in the intestines of the patients of the 2nd group the population degree of the representatives of the opportunistic microorganisms averaged 5.7 lg CFU/g (from 3 to 7 lg), and the patients of the 3rd group had 7.7 lg CFU/g (from 5.5 to 10 lg), despite a high share in the population of strains with the high and medium adhesion ability (tab. 2). At the same time the content of Lactobacillus spp. and Bifidumbacteium spp. authentically decreased ($p < 0.05$) in comparison with the indices of the reference group. So, the population degree of Lactobacillus spp. in the intestines of the patients with Alzheimer's disease and with the III dysbiosis degree made 4.48 ± 0.15 lg CFU/g, and Bifidumbacteium spp. – the patients with the II dysbiosis degree– 3.7 ± 0.2 lg CFU/g.

In particular, the average AIM values of the "intestinal" isolates of the indigenous microflora was following: Lactobacillus spp., isolated from the patients with Alzheimer's disease, having the medium adhesive potential (AIM value = 3.61 ± 0.05 bact./er.), were isolated from the patients with I and II degree of dysbiotic changes of the intestines; clinical Lactobacillus spp., isolates with the low adhesive activity (AIM value = 1.93 ± 0.03 bact./er.),were isolated from the patients with the III intestinal dysbios degree. Clinical Bifidumbacterium spp. isolates, which had their low adhesive activity (AIM value = 2.12 ± 0.03 bact./er.), were isolated from the patients with I and II degree of

dysbiotic changes of the intestines.

Table 2

Distribution of the clinically significant strains of opportunistic microorganisms, isolated from the intestines of the patients with Alzheimer's disease with their adhesive properties

Studied strains	Adhesion degree			
	low-adhesive	medium-adhesive	high-adhesive	the average AIM (adhesion index of microorganisms) (M±m)
	the number of strains (%)			
Klebsiella pneumoniae (n = 12)	0	8,3	97,1	4,6±0,4
Escherichia coli with the love enzyme activity (n = 12)	0	25,0	75,0	4,8±0,3
Proteus spp. (n = 11)	0	27,3	72,7	3,9±0,3
Cirtobacter spp. (n = 9)	11,1	22,2	66,7	3,7±0,2
Candida spp. (n = 9)	0	44,4	55,6	3,7±0,3
Enterococcus faecalis (n = 8)	0	37,5	62,5	4,1±0,2
Staphylococcus aureus (n = 6)	0	16,7	83,3	3,6±0,2
Morganella spp. (n = 5)	0	100	0	2,8±0,2
Clostridium dififcile (n = 4)	0	25,0	75,0	4,1±0,2

The analysis of studying of the quantitative level of opportunistic microflora has showed that the obtained results disclose the further prospects of the researches, directed to studying of the mechanisms of interference (replacement) of the dominant microsymbiont by opportunistic microflora in the patients with Alzheimer's disease due to the use of the personalized therapy of "Autoprobiotic".

When regarding the results of the method of delayed antagonism the following results have been obtained: the greatest efficiency of the action was registered concerning E. coli with the low enzymatic activity in Lactobacillus spp. and Bifidumbacteium spp. isolates (growth inhibition zone is 9.4 ± 0.5 mm and 5.7 ± 0.5 mm respectively); concerning Proteus spp. strains, isolated from the excrements in the quantity ≥ 6 lg CFU/g, the antagonistic action was shown by Lactobacillus spp. isolates as "++" (growth inhibition zone is 4.3 ± 0.5 mm) and as "+" Bifidumbacteium spp isolates. (growth inhibition zone is 2.1 ± 0.5 mm); concerning K.pneumoniae strains, Lactobacillus spp. isolates showed the weak level of antagonism (growth inhibition zone is 1.8 ± 0.5 mm), and among Bifidumbacteium spp. isolates 51.5% of strains did not show any antagonistic activity. Thus, the higher antagonistic activity, concerning opportunistic gram-negative representatives of Enterobacteriaceae family was shown by Lactobacillus spp.

Conclusions. 1. In the intestines of the patients of the 2nd group the population degree

of the representatives of the opportunistic microorganisms averaged 5.7 lg CFU/g (from 3 to 7 lg), and the patients of the 3rd group had 7.7 lg CFU/g (from 5.5 to 10 lg), despite a high share in the population of strains with the high and medium adhesion ability.

2. After studying of the adhesive potential of the opportunistic microorganisms, isolated from the intestines of the patients with Alzheimer's disease in clinically-significant concentration it has been determined that 64.7% of all the strains were characterized by the high level of their adhesive activity, among them there were 91.7% of K.pneumoniae, 83.3% of S.aureus, 75% of E.coli with their low enzymatic activity and C.dififcile, 72.7% of Proteus spp., 66.7% of Cirtobacter spp., 62.5% of E.faecalis, 55.6% of Candida spp.

3. So, the colonization level of the population degree of Lactobacillus spp. in the intestines of the patients with Alzheimer's disease and with the III dysbiosis degree made 4.48 ± 0.15 lg CFU/g, and Bifidumbacteium spp. – the patients with the II dysbiosis degree – 3.7 ± 0.2 lg CFU/g.

4. Critically low ($p < 0.05$) quantitative indices of Lactobacillus spp. were registered in the patients with Alzheimer's disease with the III dysbiosis degree (lg 4.48 ± 0.15 CFU/g), and Bifidumbacteium spp. – the patients with the II dysbiosis degree (lg 3.7 ± 0.2 CFU/g).

5. In vitro tests of Lactobacillus spp. and Bifidumbacteium spp., isolated from the patients with Alzheimer's disease, have not revealed any strain with a high degree of adhesion and the high "++++"level of antagonistic activity, concerning opportunistic clinical strains of E.coli, P.vulgaris, K. pneumoniae., isolated in the clinically significant concentration.

References:

1. Dzhon F. Krayan. Mikrobiom kishechnika i neyrodegenerativnyye zabolevaniya / Dzhon F. Krayan // Microbiota. Vestnik biokodeks. – 2018. – № 5. – S. 4 – 7. (In Russian)

2. Cryan J.F. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour / J.F. Cryan, T.G. Dinan // Nature Review Neuroscience. – 2012. – V. 13. – P. 701 – 712.

3. Molozhavaya O.S. Funktsii kishechnoy mikroflory organizma v norme i pri patologii / O.S. Molozhavaya, T.V. Ivakhnyuk, A.N. Makarenko A.N., R.R. Broz' // Aktual'ní problemi suchasnoï meditsini. – 2016. – T.16, Vipusk 4 (56), CH. 1.– S. 332 – 340. (In Russian)

4. Zakharov V.V. Kognitivnyye narusheniya pri neyrodegenerativnykh zabolevaniyakh / V.V. Zakharov, N.V. Vakhnina // Meditsinskiy sovet. – 2013. – № 4. – S. 30 – 35. (In Russian)

5. Microbes and Alzheimer's Disease / R.F. Itzhaki, R. Lathe, B.J. Balin, et al. Jornal of Alzheimer's Disiase. – 2016. – № 51. – P. 979 – 984.

6. Metodicheskie rekomendatsii «Mikrobiologicheskaya diagnostika disbakterioza kishechnika». – M., 2007. – 74 s. (In Russian)

7. Opredelitel' bakteriy Berdzhi. Bergey's Manual of Determinative Bacteriology : v 2 t. / kol. avt.; pod red. D. G. Khoult (biologiya), N. Krig, P. Snit, Dzh. Steyli, S. Uil'yams. – M. : Mir, 1997. (In Russian)

8. Metodika izucheniya adgezivnogo protsessa mikroorganizmov / Brilis V.I., Brilene T.A., Lentsner Kh.P i dr. // Laboratornoye delo. – 1986. – № 4. – S. 210 – 212. (In Russian)

9. Bukharin O. V. Problemy persistentsii patogenov v infektologii / O. V. Bukharin // Zhurnal mikrobiologii, epidemiologii i immunobiologii. – 2006. – S. 4 – 8.

10. Zakharova Y.V. Vliyaniye adgezivnoy aktivnosti bakteriy na ikh kolichestvennoye soderzhanie v kishechnike u VICH-infitsirovannykh detey / YU.V. Zakharova, A.A. Markovskaya // Fundamental'nye issledovaniya. – 2011. – № 7 – S. 61 – 63.

11. Ivakhniuk T.,. Molozhava O, Ivakhniuk Yu., Dovgan R., Makarenko O. Adhesive properties of lactobacillus spp. And bifidobacterium spp. Isolated from the intestines of the patients with alzheimer's disease // Modern Science. Moderní věda. – 2019. – №1. – P.139-146