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MOLECULAR GENETIC ASSESSMENT OF THE ORAL MICROBIOME IN PATIENTS WITH ALZHEIMER'S DISEASE

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According to current literature, oral microorganisms are one of the factors in the development and/or progression of Alzheimer's disease. The aim of the study was to evaluate the qualitative and quantitative composition of the microbiome of periodontal pockets of patients with Alzheimer's disease using the polymerase chain reaction. The frequency of occurrence of the main periodontal pathogens was determined and it was shown that *Tannerella forsythia* and *Fusobacterium nucleatum* were detected in 100 % of patients in this category. The main associations of periodontal pathogens were studied and it was found that in the largest number of subjects with Alzheimer's disease (66.7 %) there were associations of 4 bacteria. The quantitative composition of the oral microbiota in patients with dementia was determined and it was shown that *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* had the highest quantitative composition, and *Porphyromonas gingivalis* had the lowest. Determination of the characteristics of the oral microbiota in patients with Alzheimer's disease will clarify the mechanisms of dementia development and suggest appropriate adapted therapeutic and preventive measures to maintain oral health.

Key words: Alzheimer's disease, periodontal, generalized periodontitis, periodontal pathogens, polymerase chain reaction

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МОЛЕКУЛЯРНО-ГЕНЕТИЧНА ОЦІНКА ОРАЛЬНОГО МІКРОБІОМА В ОСІБ ІЗ ХВОРОБОЮ АЛЬЦГЕЙМЕРА

За даними сучасної літератури мікроорганізми ротової порожнини є одним з чинників розвитку та/або прогресування хвороби Альцгеймера. Метою роботи було оцінити якісний і кількісний склад мікробіому пародонтальних кишень осіб з хворобою Альцгеймера за допомогою полімеразно-ланцюгової реакції. Було визначено частоту зустрічаємості основних пародонтопатогенів і показано, що у 100 % пацієнтів даної категорії виявляється *Tannerella forsythia* і *Fusobacterium nucleatum*. Досліджено основні асоціації пародонтопатогенів та встановлено, що у найбільшого числа обстежених осіб з хворобою Альцгеймера (66,7 %) зустрічалися асоціації 4 бактерій. Визначено кількісний склад оральної мікробіоти у пацієнтів з деменцією і показано, що найбільший кількісний склад мали *Aggregatibacter actinomycetemcomitans* та *Fusobacterium nucleatum*, найменший – *Porphyromonas gingivalis*. Визначення особливостей оральної мікробіоти в осіб з хворобою Альцгеймера дозволить уточнити механізми розвитку деменції та запропонувати відповідні адаптовані лікувально-профілактичні заходи для збереження здоров'я ротової порожнини.

Ключові слова: хвороба Альцгеймера, пародонт, генералізований пародонтит, пародонтопатогени, полімеразно-ланцюгова реакція

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The incidence of dementia and its prevalence among the world's population continue to increase [15]. Alzheimer's disease (AD) is the most common form of dementia (60–80 %) [1]. According to the WHO, there are currently around 35.6 million people worldwide affected by AD, and this number will double by 2030, reaching a total of 131.8 million people worldwide, and triple by 2050. The WHO reports that more than $600 billion a year is spent on treating and caring for people with dementia, and this figure is set to grow, as senile dementia is called a "ticking time bomb" [6].

Recently, the results of a study conducted at the Chebotarev Institute of Gerontology of the National Academy of Medical Sciences of Ukraine showed that the overall prevalence of dementia in people aged 60 is 10.4 %. This figure doubles every 5 years, meaning that 45 % of the Ukrainian 80-year-old population suffers from dementia, and AD causes cognitive disorders in 60–70 % of cases [5]. The brain's "gray matter" deterioration results in memory loss and dementia due to ß-amyloid accumulation. Researchers are split: some emphasize tau protein's importance, while others focus on ß-amyloid and its variants in the disease's progression [2].

Today, the question remains: why does Alzheimer's disease occur? There are several theories that try to explain the development of the disease. The causes of Alzheimer's disease include hereditary

predisposition, atherosclerosis, oxygen deficiency, beta-amyloid deposition in brain cells, decreased acetylcholine levels, chromosomal abnormalities, specific immune reactions, exposure to toxic substances, trauma (psychological, craniocerebral), and viruses. Many researchers include slow onset depression, high blood pressure, low intelligence, diabetes mellitus, etc. as risk factors for the disease. [3, 8]. In recent years, the scientific community has increasingly begun to talk about the infectious origin of Alzheimer's disease. Scientists have discovered the causative agent of periodontitis, Porphyromonas gingivalis, in the brains of people who died of Alzheimer's disease. This was not the first time that these two factors were interrelated [13, 14]. Bacteria linked to periodontitis, like P. gingivalis, can influence the development of AD in certain individuals. P. gingivalis releases enzymes and proteins that damage tissues and spark inflammation. Its outer membrane vesicles, which can spread throughout the body, might connect periodontitis to AD neurodegeneration. These vesicles can breach the blood-brain barrier, exacerbating AD-specific issues like neuroinflammation, plaque formation, and iron imbalance, leading to neuronal death [11].

Some researchers believe oral infections trigger immune responses, releasing cytokines into the blood, which affect the blood-brain barrier. Studies indicate that this allows periodontal pathogens to reach the central nervous system, prompting the brain to form defensive plaques. However, whether the relationship between AD and periodontitis, taking into account periodontal pathogens, is causal remains an open question [7], which necessitated the need for research.

The purpose of the study was to examine the quantitative and qualitative composition of periodontal pathogens in the contents of periodontal pockets of patients with Alzheimer's disease using the polymerase chain reaction.

Materials and methods. A total of 27 patients with Alzheimer's disease aged 62–80 years were examined. The distribution of patients by gender was uneven: the vast majority of the patients were women (21 people or 77.8 %), the number of men was 3.5 times less (22.2 %). Examination of patients with AD and sampling of material for further molecular genetic studies was carried out at the State Institution "D.F. Chebotarev Institute of Gerontology of the National Academy of Medical Sciences of Ukraine", Kyiv (according to the Agreement on Scientific Cooperation of 25.08.2022). All clinical manipulations were performed after signing an informed consent to the examination and sampling of biological material either by the patient or by the guardian.

The gingival fluid was collected from the periodontal pocket for the determination of periodontal pathogenic microflora by polymerase chain reaction (PCR) using sterile paper endodontic pins. The samples were placed directly into tubes with the "DNA-EXPRESS" reagent (SPC "Litech").

The presence and quantitative content of the following pathogens was analyzed: Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Porphyromonas endodontalis (Pe), Treponema denticola (Td), Tannerella forsythia (Tf), Prevotella intermedia (Pi), Fusobacterium nucleatum (Fn).

Amplification was performed on a CFX96 device (Bio-Rad, USA) using the Fluoropol kit (SPC "Litech"), and the fluorescent signal was recorded using 2 channels – FAM/ROX and HEX.

The obtained results were subjected to statistical processing in Microsoft Excel 2010 and Statistica 6.1 (StatSoftInc., Serial No. AGAR909E415822FA) under the Windows XP operating system. Statistically significant differences were evaluated using Student's t-test [4].

Results of the study and their discussion. The study of the oral microbiota in patients with AD is necessary to identify the peculiarities of its qualitative and quantitative composition in order to develop adapted individualized schemes for the prevention of dental pathology, taking into account the literature on the involvement of the oral microbiota in the pathogenesis of dementia, including AD.

Clinical and laboratory examination of patients with AD showed a 100 % prevalence of periodontal disease in the form of generalized periodontitis (GP). The distribution of patients according to the severity of GP was as follows: the number of patients with GP I–II, II degree was 55.6 % (15 people, including 14 women and 1 man), with GP II–III degree – 44.4 % (12 people, including 3 women and 1 man). The number of women with AD, which exceeded the number of men by 3.5 times, can be explained by the fact that the average life expectancy of women in Ukraine, according to official data, is 78 years, while men are 68 years old (United Nations Population Fund report). And since Alzheimer's disease is most common in people aged 65 and older (although the manifestation of the disease can occur at a much younger age), the percentage of men among the elderly is very low.

The qualitative and quantitative composition of the oral microbiome was studied using the polymerase chain reaction.

The results of microbiological examination of the periodontal pockets of patients with AD indicate a high degree of contamination with periodontal pathogens. Thus, Tannerella forsythia (Tf) and

Fusobacterium nucleatum (Fn) were found in 100 % of the examined patients. Porphyromonas endodontalis was the second most frequent bacterium (77.8 %). The frequency of Porphyromonas gingivalis was 44.4 %. The lowest colonization of periodontal pockets among the studied periodontal pathogens in patients with AD was in Aggregatibacter actinomycetemcomitan (Aa), Treponema denticola (Td) and Prevotella intermedia (Pi) (33.3 % each) (Fig. 1).

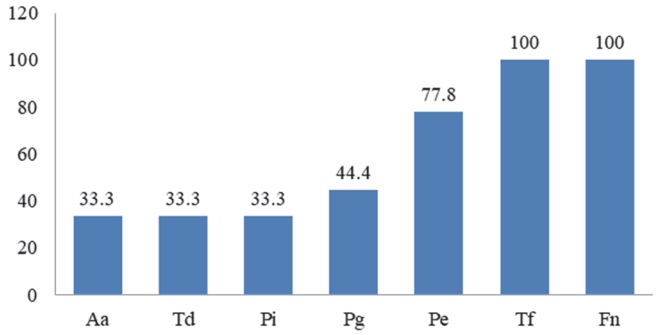

Fig. 1. Frequency of periodontal pathogens in patients with Alzheimer's disease, %.

According to Socransky S. (1998), who formulated the so-called classification of periodontal microbial complexes, which consist of periodontopathogenic micro-organisms with factors of virulence and pathogenicity against periodontal tissues, only Tannerella forsythia was isolated from periodontopathogens of the "red" complex in all examined patients. Fusobacterium nucleatum, which was also isolated in 100 % of the examined patients, belongs to the "orange" complex.

Periodontal pathogens are usually not detected separately, but in the form of associations or in combination with other pathogens.

The following data were obtained in the study of microbial associations in the examined persons. There were no monobacteria or associations of 2 bacteria in the examined patients. Associations of 3 periodontal pathogens were observed in 3 patients, which amounted to 11.1 % (Table 1).

Table 1

Characterization of associations of periodontal pathogens in patients with Alzheimer's disease

Number of periodontal pathogens in the association	Frequency of occurrence of associations	
	abs.	%
Associations of 3 bacteria	3	11.1
Associations of 4 bacteria	18	66.7
Associations of 5 bacteria	3	11.1
Association of 6 bacteria	3	11.1

The associations of 3 bacteria were represented by Porphyromonas endodontalis/Tannerella forsythia/Fusobacterium nucleatum.

The largest number of patients with AD (66.7 %) had associations of 4 bacteria. The most frequent association was Porphyromonas gingivalis/Porphyromonas endodontalis/Tannerella forsythia/Fusobacterium nucleatum (29.7 % of cases). The associations had almost the same frequency of occurrence (11.1–14.8 %):

– Porphyromonas gingivalis/Treponema denticola/Tannerella forsythia/ Fusobacterium nucleatum;

– Porphyromonas endodontalis/Treponema denticola/Tannerella forsythia/ Fusobacterium nucleatum;

– Porphyromonas endodontalis/Prevotella intermedia/Tannerella forsythia/ Fusobacterium nucleatum;

– Aggregatibacter actinomycetemcomitans/Prevotella intermedia/Tannerella forsythia/ Fusobacterium nucleatum.

In another 6 patients with AD, associations of 5 and 6 periodontal pathogens were detected (11.1 %, respectively). In the association of 5 bacteria among the 7 periodontal pathogens studied, Porphyromonas gingivalis and Treponema denticola were absent, and in the association of 6 bacteria – Prevotella intermedia.

As for the quantitative composition of periodontal pocket microorganisms in patients with AD, the following results were obtained (Table 2).

Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum ($x10^5$) had the highest number of species, and Porphyromonas gingivalis ($x10^3$) had the lowest.

It is worth noting that the presence of Porphyromonas gingivalis in the contents of periodontal pockets in patients directly correlated with the severity of generalized periodontitis and the depth of periodontal pockets. Thus, among patients with generalized periodontitis of II–III severity and severe periodontal pockets, Porphyromonas gingivalis was detected in 100 %. Among patients with generalized

periodontitis of I–II, II degree or in patients with severe gingival recession, Porphyromonas gingivalis was detected only in 18.5 %.

Among the patients with Treponema denticola, patients with generalized periodontitis of II–III degree prevailed (66.7 %), the proportion of patients with generalized periodontitis of I–II, II degree was 33.3 %.

Table 2

Quantitative composition of microorganisms in the contents of periodontal pockets of patients with AD

Type of microorganism	Quantity
Aggregatibacter actinomycetemcomitans	$3.1\pm1.23x10^5$
Porphyromonas gingivalis	$5.5\pm1.1x10^3$
Porphyromonas endodontalis	$3.0\pm1.1x10^4$
Treponema denticola	$2.3\pm0.8x10^4$
Tannerella forsythia	$4.0\pm1.6x10^4$
Prevotella intermedia	$6.7\pm1.3x10^4$
Fusobacterium nucleatum	$3.8\pm1.8x10^5$

As for Aggregatibacter actinomycetemcomitans, a representative of the “green” complex, other data were obtained. Among the patients with the isolated microorganism, patients with generalized periodontitis of I–II, II degree prevailed (75 %), the proportion of patients with more severe periodontitis was 25 %.

There was no correlation between Porphyromonas endodontalis and the severity of the dystrophic-inflammatory process in the periodontium. Its highest quantitative values were obtained in a patient with generalized periodontitis of I–II, II degree ($4.4x10^4$ units), the next ($3.2x10^4$ units) in a patient with GP of II–III degree of severity.

As for the microorganism of the “orange” complex Prevotella intermedia, all patients in whom it was isolated had generalized periodontitis of I–II, II degree. In patients with more severe periodontal disease, this microorganism was not detected in sufficient quantities for identification.

Thus, during the studies to assess the microbial contamination of periodontal pockets of patients with AD by polymerase chain reaction, it was found that Fusobacterium nucleatum (100 %) had the highest frequency of occurrence among the examined category of patients, which correlates with the data of Zixin Fan et al., 2023 [9], and Tannerella forsythia (100 %), which confirms the data of Leblhuber F. et al., 2020 [12] on the alleged involvement of these periodontal pathogens in the pathophysiological and immunoinflammatory mechanisms of Alzheimer's disease.

The periodontal pathogen Porphyromonas gingivalis, which has recently been the subject of many publications in the modern literature as a possible cause of AD onset or progression, was found only in 44.4 % of the examined subjects, and its frequency directly correlated with the severity of the dystrophic-inflammatory process in the periodontium

Several putative mechanisms have been described that could explain how the oral microbiota affects the homeostasis of the central nervous system, including Diffusion of oral microorganisms into the bloodstream with subsequent inflammatory response associated with cerebrovascular atherosclerosis; blood-brain barrier (BBB) breakdown; migration of oral bacteria through the peripheral trigeminal nerve endings to the trigeminal ganglion and then to the brain; migration of microorganisms via the lymphatic circulation. Although these theories explain the possible pathway of migration of oral microbiota to the brain, it is still unknown what contributes most to AD. With increasing age, BBB permeability increases in elderly patients. As a result, pathogenic microorganisms can easily cross the BBB and penetrate the brain tissue, directly affect neurons, activate an inflammatory cascade reaction, and cause direct damage to the central nervous system. In addition, the oral microbial population indirectly affects AD by releasing bacterial toxins, outer membrane vesicles, and proinflammatory factors that enter the brain with blood [9].

AD is a major subtype of dementia and causes impaired memory, thinking, and communication skills in people who have behavioural and psychological symptoms such as depression and aggression [8]. It is very important for them to maintain their oral health, as oral health is an important and integral part of their overall health. Neglecting oral health leads to the development of dental diseases that are difficult and expensive to treat.

However, in order to maintain oral health in this population, especially in the early stages of AD, older adults should maintain daily oral hygiene to prevent the need for extensive and complex procedures. However, maintaining oral health becomes difficult when AD progresses to middle and late stages due to

loss of interest in oral care, so guidelines for oral health maintenance become necessary, as the methods used to provide this support differ from older adults, especially those with AD [10, 12].

The results of the study of the qualitative and quantitative composition of the oral microbiota can be the basis for the development of personalized therapeutic and prophylactic complexes for oral care at home with the inclusion of pathogenetically based components of antimicrobial action.

Conclusions

1. Based on the results of the evaluation of periodontal pockets in patients with Alzheimer's disease using the polymerase chain reaction, the frequency of occurrence of the main periodontal pathogens was determined. It has been shown that Tannerella forsythia and Fusobacterium nucleatum are detected in 100 % of patients in this category.

2. In patients with AD, the main associations of periodontal pathogens were studied and it was found that in the largest number of patients with AD (66.7 %) there were associations of 4 bacteria with the most frequent association of Porphyromonas gingivalis / Porphyromonas endodontalis / Tannerella forsythia / Fusobacterium nucleatum (29.7 %).

3. The quantitative composition of the oral microbiota in patients with AD was determined, it was shown that Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum ($x10^5$) had the largest quantitative composition, Porphyromonas gingivalis had the smallest ($x10^3$).

4. A direct correlation between the detection of Porphyromonas gingivalis and the severity of the dystrophic-inflammatory process in the periodontium and the presence of deep periodontal pockets was shown. Treponema denticola occurs mainly in patients with generalized periodontitis of II–III degree (66.7 %), Aggregatibacter actinomycetemcomitans – in patients with generalized periodontitis of I–II, II degree (75 %).

5. Determining the characteristics of the oral microbiota in patients with Alzheimer's disease will clarify the mechanisms of dementia development and offer appropriate adapted therapeutic and preventive measures to maintain oral health.

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