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Dynamics of *Nakaseomyces glabratus* biofilm formation

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ABSTRACT

Formation of biofilms of microorganisms, including those of *Nakaseomyces glabratus*, is responsible for the development of local and systemic pathologies in humans and animals. The system of gene expression coordination (quorum sensing) in the representation of signaling molecules allows regulation of the amount and composition of biofilm populations thus expanding the adaptive capacity of microorganisms. In the presence of gingivitis and odontolithiasis clinical signs in dogs, excessive growth of gram-positive yeast microorganisms is a differential sign of the decreased resistance of the digestive system mucous membranes to colonization. Examination of the densitometric and morphometric parameters revealed general patterns of biofilm formation, regardless of the source of *Nakaseomyces glabratus* isolates. Depending on the time of cultivation of the microorganisms, a gradual increase in the optic density absolute values was established. Intercellular communications were achieved by coaggregation of the heteromorphic structures, which formed clusters with rounded liquid-containing formations detected among them. The population immobilization of the architectonics of the mature three-dimensional biofilm, as consistent with cultivation conditions, was accompanied by the differentiation of numerous cells of different sizes and shapes depending on the stage of the cell cycle. Results of the examination of the general patterns of the heterogeneous micromycete population development are promising for expanding the boundaries of knowledge of the adaptation mechanisms of ubiquitous microorganisms to long-term *in vivo* and *in vitro* persistence. Methods for studying morphometric and densitometric indicators avoiding interfering into the natural biofilm architectonics are recommended to optimize the long-term and retrospective mycological studies, as well as to develop effective mycosis treatment and prevention regimens.

Keywords: biofilms, microfungi, *Nakaseomyces glabratus*, optic density, microscopy, differential signs

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Динамика развития биопленок грибов *Nakaseomyces glabratus*

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РЕЗЮМЕ

Формирование биопленок микроорганизмов, в том числе и *Nakaseomyces glabratus*, обусловливает развитие локальных и системных патологий человека и животных. Система координации экспрессии генов (quorum sensing) при репрезентации сигнальных молекул позволяет регулировать количество и состав популяций биопленок, что расширяет адаптивный потенциал микроорганизмов. При наличии клинических признаков гингивита и одонтолитиаза у собак избыточный рост грамположительных дрожжевой формы микроорганизмов является дифференциальным признаком снижения колонизационной резистентности слизистой оболочки пищеварительной системы. Исследование денситометрических и морфометрических показателей выявило общие закономерности развития биопленок, независимо от источника выделения изолятов *Nakaseomyces glabratus*. В зависимости от времени культивирования микроорганизмов установили постепенное увеличение значений абсолютных величин оптической плотности. Реализация межклеточных коммуникаций достигалась коагрегацией гетероморфных структур, формирующих кластеры, между которыми выявлялись округлые образования, содержащие жидкость. Популяционная иммобилизация архитектоники зрелой трехмерной биопленки, в соответствии с условиями культивирования, сопровождалась дифференциацией многочисленных клеток разных размеров и форм в зависимости от стадии клеточного цикла. Результаты исследований общих закономерностей развития гетерогенной популяции микромицетов представляют перспективность для расширения границ познания механизмов адаптации убиквитарных микроорганизмов к длительной персистенции *in vivo* и *in vitro*. Способы изучения морфометрических и денситометрических

показателей биопленок без нарушения естественной архитектоники рекомендуются для оптимизации микологических исследований, являющихся длительными и ретроспективными, а также разработки эффективных схем лечения и профилактики микозов.

Ключевые слова: биопленки, микроскопические грибы, *Nakaseomyces glabratus*, оптическая плотность, микроскопия, дифференциальные признаки

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INTRODUCTION

Nakaseomyces glabratus microfungi (formerly *Candida glabrata*) are recognized as one of the priority pathogens for research that cause development of nosocomial infections with high epidemiological mortality rates [1]. With the development of septicemia, endocarditis, pyelonephritis, bronchopneumonia, catheter-, prosthesis-associated pathologies, *N. glabratus* isolates are significantly often characterized by multidrug resistance [2, 3, 4].

The tendency of a statistically significant increase in the etiological significance of *N. glabratus* has been established with the development of opportunistic endogenous infection of animals against the antibiotic therapy [5, 6, 7, 8, 9, 10].

With the development of micromycete overgrowth syndrome, the pathogenic potential is realized by increasing the biofilm biomass, which are a heterogeneous association of microorganisms aggregated by the extracellular matrix [11, 12, 13].

Gene expression coordination system (quorum sensing), when representing signaling molecules, allows regulation of the number and composition of biofilm populations thus expanding adaptive potential of the microorganisms [12, 14, 15].

Transcriptional control of adhesion, invasion, and synthesis of polymeric substances allows the multicellular population to realize virulent properties in interaction with immunocompetent cells, provides protection against phagocytosis and effects of chemotherapeutic drugs and disinfectants [16, 17, 18].

Initiation, development and outcome of superficial, deep and systemic mycoses with excessive growth and increase in pathogenic potential of ubiquitous microorganisms are attributed to hyperaggregation, presence of dissociative variants and dispersion of heterogeneous biofilms. The investigation of the ways to indicate biofilms, including when exposed to chemotherapeutic drugs and disinfectants, in the future will allow the development of fungicidal medicinal products aimed to block the synthesis or destruction of the biofilm extracellular matrix.

To improve the procedures of mycological research and development of preventive antiepidemic measures, the priority area of scientific investigations is to expand the boundaries of knowledge of the mechanisms of the multi-stage biofilm formation process.

The aim of the work was to study the morphometric and densitometric parameters of *N. glabratus* isolates recovered and identified during the progress of gingivitis and odontolithiasis in dogs.

MATERIALS AND METHODS

Strains. N. glabratus isolates were used in the experiments, which were recovered from the swabs and scrapings of the oral mucosa of dogs demonstrating gingivitis and odontolithiasis clinical signs. Reference strain ATCC 66032 was tested as control [19].

Nutrient media. Meat-extract broth (HiMedia Laboratories Pvt. Ltd., India), bovine blood serum (Microgen, Russia); rice extract agar (API-System R.A.T., France); Sabouraud glucose agar with penicillin and streptomycin (100 IU/L); Columbia agar; chromogenic agar (BioMedia, Russia).

Test-kits. HiCandida™ Identification Kit (HiMedia Laboratories Pvt. Ltd., India) was used to identify the microorganisms.

Indication and identification of microorganisms. For microscopy, fingerprints were prepared from the swabs and scrapings and Gram-stained.

Medium quality control – sterility test at (36 ± 1) °C for 48 hours.

To indicate hyphal germ tubes, the day-old cultures of the microorganisms were cultured in meat-extract broth supplemented with 1.0 mL of blood serum at (35 ± 2) °C for 5 hours and microscopic examination of the methylene blue-stained specimens was performed.

Chlamydospore presence was tested on native microorganism culture specimens grown on rice agar at (25 ± 2) °C for 24 hours.

When adding cycloheximide (0.5 g/L of the medium) to Sabouraud agar, the growth of microorganisms at (25 ± 2) °C during 72 hours was recorded.

The microorganisms were indicated and identified with due consideration of the typical growth properties of the microorganisms and using generally accepted methods [20, 21].

To record the biochemical properties, the day-old cultures of the microorganisms (optical density OD = 0.5; wavelength 620 nm) were added into the wells of HiCandida™ Identification Kit panel and cultivated at (22.5 ± 2) °C for 48 hours. The microorganisms were identified in accordance with the identification tables and codes of the specified test-kit.

Investigation of the biofilm formation dynamics. To record the biofilm formation dynamics, the microorganisms were cultivated under static conditions at (35 ± 2) °C for 18, 24 and 48 hours.

To assess the densitometric parameters, upon the specified cultivation period completion, the fluid was removed and the sediment was washed three times with 200.0 µL of the phosphate buffer solution (pH 7.2). The specimens were fixed with 150.0 µL of 96° ethanol for 15 minutes. The specimens were further dried at (35 ± 2) °C for 20 minutes, 0.5% crystalline violet solution was added and transferred to the thermostat at (35 ± 2) °C for 5 minutes. The contents of the wells were removed, the wells were washed three times with 200.0 µL of the phosphate buffer solution (pH 7.2) and dried. The stain was eluted with 200.0 µL of 96° ethanol for 30 min [13].

The OD of the tested specimens was determined by the degree of crystalline violet binding (HiMedia Laboratories Pvt. Ltd., India) using a photometric analyzer Immuno-Chem-2100 (High Technology Inc., USA) at wavelength 580 nm (OD_{580}).

For morphometric tests, the slides were placed in Petri dishes and 100.0 mL of 18-hour cultured microorganism suspension were added at 10^5 CFU/mL. After a predetermined time of the microorganisms' cultivation, the specimens were fixed three times, sequentially immersed in 96° ethanol for 10 minutes and air-dried for 10 minutes. Then the specimens were stained with gentian violet solution supplied with the Gram staining kit (BioVitrum, Russia).

During representative sampling of significant frequency of occurrence (≥ 90.0% of the field of Carl ZEISS Axio Lab.A1 optical microscope, Germany) microphotography was made using ADF PRO 08 digital camera (China) with matrix resolution of 8 megapixels (4K).

The resulted data were processed by statistical analysis method using the Student's criterion, the results were considered reliable at $p < 0.05$ [18].

RESULTS AND DISCUSSION

Indication and identification of microorganisms. With gingivitis and odontolithiasis clinical signs in dogs, intense yellow-gray deposits tightly attached to the mucous membrane were observed. Bright red ulcers were generally detected when these deposits were removed.

Optical microscopy of fingerprints of oral mucosa scrapings revealed a large number of gram-positive yeast-shaped microorganisms of (1.1–2.1 × 3.1–4.0) µm in size.

After 48 hours at (35 ± 2) °C, the microorganisms formed shiny white colonies on Sabouraud agar.

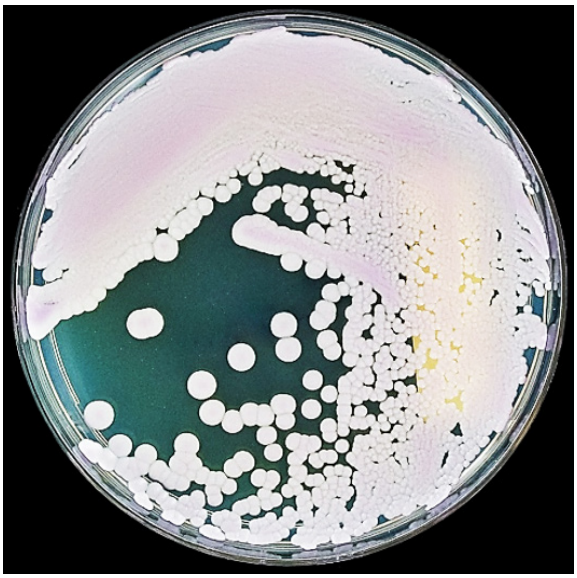

Fig. 1. Features of N. glabratus growth on chromogenic agar at (35 ± 2) °C for 48 hours

Presence of chromogenic substrates in the indicator medium allowed differentiation of *N. glabratus* colonies of pink color with a slightly purple hue (Fig. 1).

Nakaseomyces glabratus microfungi fermented maltose, trehalose; they did not ferment urease, melibiose, lactose, sucrose, galactose and xylose. The microorganisms did not have any urease activity.

Microscopic examination of the specimens of the microorganisms cultured in 1 mL serum supplemented-meat-extract broth at (35 ± 2) °C for 5 hours did not reveal formation of hyphal germ tubes; therefore, the test result was negative.

In microorganism cultures grown on rice agar at (25 ± 2) °C for 24 hours, chlamydospores were not detected; therefore, the test result was negative.

When incubating the culture on cycloheximide-containing Sabouraud agar (0.5 g/L) at (25 ± 2) °C for 72 hours, no microorganisms' growth was observed; therefore, the test result was negative.

When recording the microorganisms' tolerance to temperature (35 ± 2), (42 ± 2), (45 ± 2) °C for 24 hours, typical turbidity of the liquid Sabouraud medium, presence of a slight sediment and gray thin film on the surface of the medium were detected; therefore, the test result was positive.

The reference strain and isolates, regardless of the source of isolation, demonstrated properties typical of yeast-like fungi of *N. glabratus* species (Table).

Table
Differential features of *N. glabratus*

Microorganism cultures	Differential signs					
	Hyphal tubes	Chlamydospores	Cycloheximide	Temperature tolerance		
				(35 ± 2) °C	(42 ± 2) °C	(45 ± 2) °C
N. glabratus, ATCC 66032	–	–	–	+	+	+
N. glabratus, swabs from the dorsal surface of the tongue	–	–	–	+	+	+
N. glabratus, swab from the gums	–	–	–	+	+	+

"+" – presence of microbial growth; "–" – absence of microbial growth.

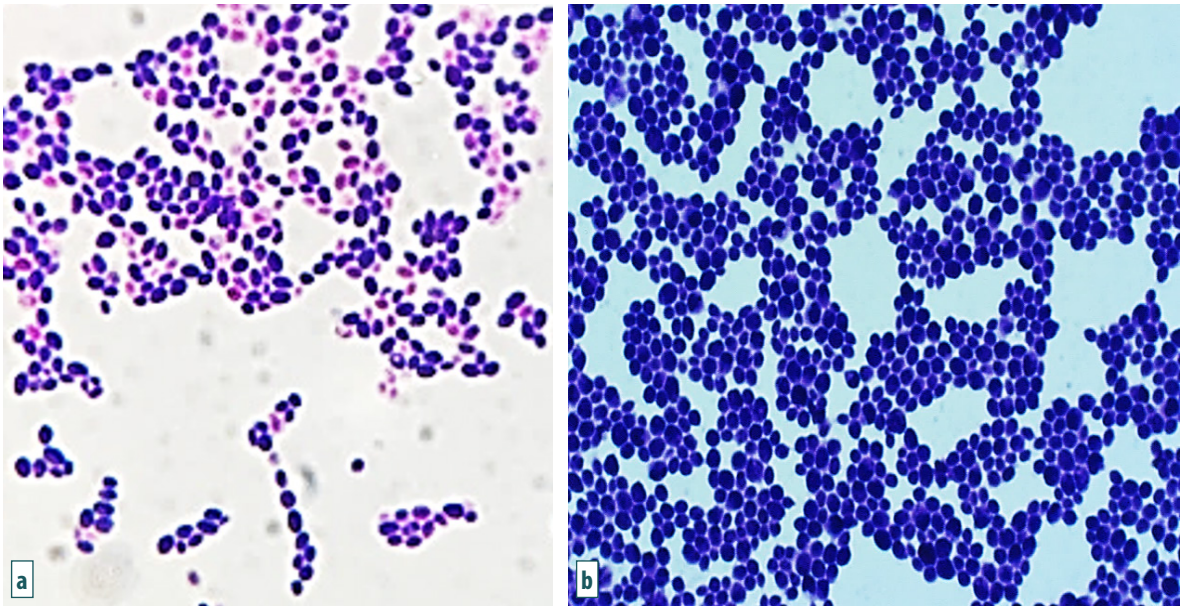

Fig. 2. Stages of N. glabratus biofilm formation at (35 ± 2) °C in beef-extract broth (yeast-like cells of species-typical shape and size aggregated by the extracellular matrix): a – in 18 hours; b – in 24 hours. Methylene blue staining, ocular lens 10×, objective lens 100×, immersion

Biofilm formation dynamics. The study of densitometric and morphometric parameters revealed common patterns of biofilm formation of *N. glabratus* isolates, regardless of the source of isolation.

Depending on the time of cultivation of the microorganisms, a gradual increase in the OD absolute values of the tested specimens was established: after 18 hours – from 0.218 ± 0.05 to 0.221 ± 0.08, the intensity of biofilm formation – ≥ 0.1; after 24 hours – from 0.289 ± 0.04 to 0.297 ± 0.09, the intensity of biofilm formation – ≥ 0.2; after 48 hours – from 0.331 ± 0.10 to 0.350 ± 0.08, the intensity of biofilm formation – ≥ 0.3.

During representative sampling according to the morphometric parameters of the significant frequency of occurrence (≥ 90.0% in the field of view of an optical microscope – yeast cells of shape and size typical for *N. glabratus* species were found aggregated by the extracellular matrix (Fig. 2).

Depending on the time of cultivation, such stages as adhesion, fixation, coagulation, microcolonies, and dispersion were revealed during the biofilm formation.

At the early stages of the formation, primary attachment due to planktonic forms sorption, i.e. adhesion of the microorganisms to the test substrate, was reported (in our studies – to the slide surface). This stage is considered reversible, that is, the attached cells can detach from the substrate and return to the planktonic form.

During the adhesion, the cell walls of the microorganisms produced exocellular molecules that ensured the cell fixation to the slide surface. The cells tightly fixed to the substrate contributed to the adhesion of the subsequent cells.

The population immobilization of the architectonics of the mature three-dimensional biofilm dependent on the cultivation conditions is mediated by the quorum

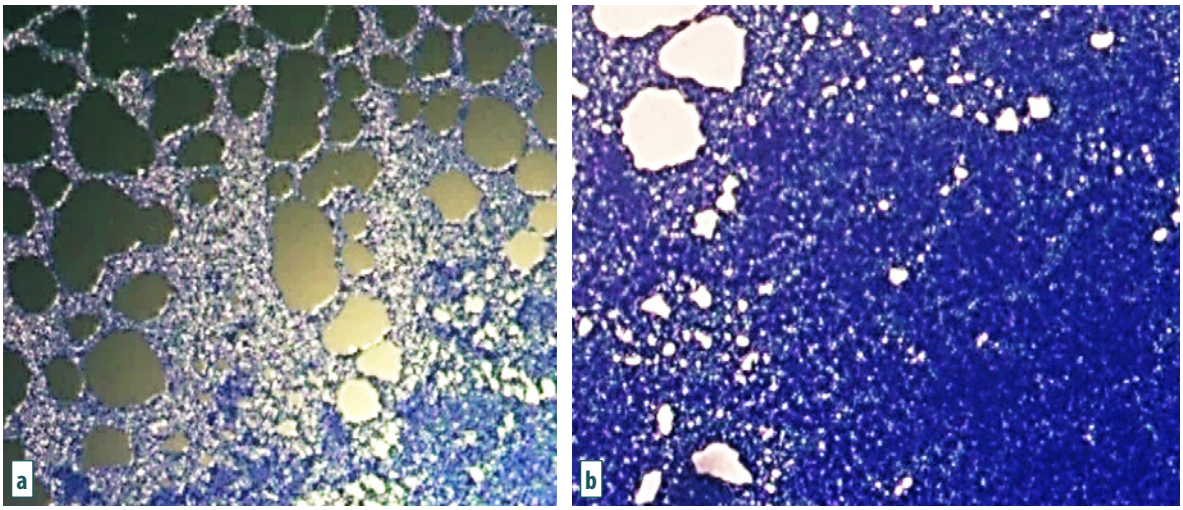

Fig. 3. Stages of N. glabratus biofilm formation at (35 ± 2) °C in beef-extract broth in 48 hours: intense cell proliferation is accompanied by the formation of net-like structures and extracellular matrix thickening. Gentian violet staining: a – ocular lens 10×, objective lens 5×; b – ocular lens 10×, objective lens 10×

sensing phenomenon. This is a special form of extracellular communication of the microorganisms due to the synthesis of numerous exocellular molecules, whose concentration is proportional to the cell density.

When increasing the cultivation time, intensive proliferation was accompanied by differentiation of yeast forms and formation of an extracellular matrix (Fig. 3).

The number of the attached dividing cells significantly increased and, accordingly, the growth of microcolonies was observed, which resemble colonies formed on dense nutrient media. With an increase in the number of microorganisms due to an increase in the synthesis of exocellular molecules, extracellular bonds were formed, and the population, called a mature biofilm, was immobilized.

When certain sizes of microcolonies were reached, dispersion of individual cells periodically occurred, which were after a while able to attach to the surface and form a new microcolony.

The general pattern of the regularity and compactness of the multicellular heteromorphic biofilm population was determined by the cell cycle stages and degree of development of the extracellular matrix. The cellular composition of the mature biofilm was represented by ovoid- or ellipsoid-shaped cells of typical size of (1.1–1.9) × (3.1–3.4) microns (Fig. 4).

The population immobilization of the architectonics of the mature three-dimensional *N. glabratus* biofilm, as consistent with cultivation conditions, was accompanied by the differentiation of numerous cells of different sizes and shapes depending on the cell cycle stage [12, 15, 18].

Results of the examination of the general patterns of the heterogeneous microorganism population development are promising for expanding the boundaries of knowledge of the adaptation mechanisms of ubiquitous microorganisms to long-term *in vivo* and *in vitro* persistence.

Methods for studying morphometric and densitometric indicators avoiding interfering into the natural biofilm architectonics are recommended to optimize the long-term and retrospective mycological studies, as well as to develop effective mycosis treatment and prevention regimens.

CONCLUSION

Microscopy of the fingerprints of the scrapings of the oral mucosa of dogs demonstrating gingivitis and odontolithiasis clinical signs revealed excessive growth of gram-positive yeast microorganisms. Depending on the time of cultivation of the microorganisms, a gradual increase in the OD absolute values was established. Extracellular communications were achieved by coaggregation of the heteromorphic structures forming clusters, among which rounded liquid-containing formations were detected. Regularity and compactness of the multicellular heteromorphic population of mature biofilm is determined by the cell cycle stages and degree of the extracellular matrix development.

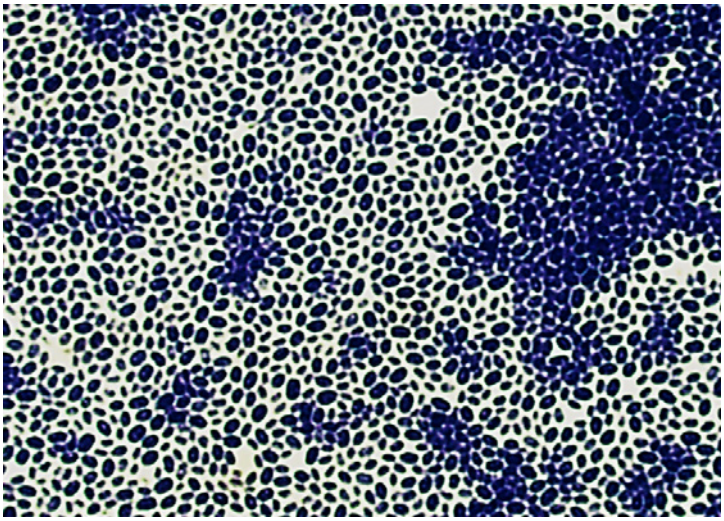

Fig. 4. Stage of N. glabratus biofilm formation at (35 ± 2) °C on beef-extract broth in 48 hours: regularity and compactness of multicellular heteromorphic population of the mature biofilm. Gentian violet staining, ocular lens 10×, objective lens 100×, immersion

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