BY-NC-SA

doi: 10.31146/2949-4664-apps-2-2-5-11

Secretory inhibitor of lysozyme and biofilm formation of intestinal strains *Candida* in children with reactive arthritis

O. V. Bukharin[1], N. B. Perunova[1], E. V. Ivanova[1], O. E. Chelpachenko[1], A. I. Khavkin[2, 3]

[1] Institute of Cellular and Intracellular Symbiosis, Ural department, Russian Academy of Science, Laboratory of Biomonitoring and Molecular-Genetic research, Orenburg, 29, Naberezhnaya str., 460014, Russia

[2] Research Clinical Institute of Childhood, Ministry of Health care of the Moscow Region, 62, Bolshaya Serpukhovskaya str, Moscow, 115093 Russia

[3] Federal State Autonomous Educational Institution of Higher Education "N. I. Pirogov Russian National Research Medical University" of the Ministry of Health of the Russian Federation, 2, Taldomskaya St., Moscow, 125412, Russia

Summary

This short communications compares the phenotypic properties of *Candida* species isolated from gut of children with reactive arthritis, such as secretory inhibitor of lysozyme and biofilm formation.

A total of 65 clinical strains of *Candida* species isolated from the feces of healthy children and children with ReA were tested. SIL production of *Candida* by inhibiting lysozyme activity against *Micrococcus luteus ATCC 15307* were studied (mg/mL*OD). Quantitative assessment of biofilm formation was performed using crystal violet binding assay method (CU). A significantly higher proportion of ReA strains were SIL-positive (77.8% vs. 40.0%) and BF-positive (64.4% vs. 30.0%) compared with non-ReA strains ($P < 0.01$).

These results suggest that the functional significance of SIL production and biofilm formation must be estimated, of investigating the roles of intestinal of *Candida* in the pathogenesis of spondylarthritis, including reactive arthritis. It is imperative to delineate microbial factors that contribute to ReA development.

Corresponding author:
Natalia B. Perunova
e-mail:
perunovanb@gmail.com

EDN: ZNKQMS

Keywords: *Candida albicans*, phenotypic properties, reactive arthritis

Передовая статья

Секреторный ингибитор лизоцима и биопленкообразования кишечных штаммов *Candida* у детей с реактивным артритом

Бухарин О. В.[1], Перунова Н. Б.[1], Иванова Е. В.[1], Челпаченко О. Е.[1], Хавкин А. И.[2, 3]

[1] Институт клеточного и внутриклеточного симбиоза УрО РАН, Россия, 460014, г. Оренбург, ул. Набережная, 29

[2] Государственное бюджетное учреждение здравоохранения Московской области «Научно- исследовательский клинический институт детства Министерства здравоохранения Московской области», 115093, г. Москва, ул. Большая Серпуховская, 62, Российская Федерация

[3] Федеральное государственное автономное образовательное учреждение высшего образования «Российский национальный исследовательский медицинский университет имени Н. И. Пирогова» Министерства здравоохранения Российской Федерации, 125412, Москва, ул. Талдомская, 2

Для переписки:
Перунова Наталья Борисовна
e-mail:
perunovanb@gmail.com

Резюме

В представленном кратком сообщении сравниваются фенотипические свойства видов *Candida*, выделенных из кишечника детей с реактивным артритом, такие как секреторный ингибитор лизоцима и образование биопленок.

Всего протестировано 65 клинических штаммов видов *Candida,* выделенных из фекалий здоровых детей и детей с РеА. Изучали продукцию SIL Candida путем ингибирования активности лизоцима против Micrococcus luteus ATCC 15307 (мг/мл*ОД). Количественную оценку образования биопленок проводили с использованием метода анализа связывания кристаллического фиолетового (CU). Значительно более высокая доля штаммов РеА была SIL-положительной (77,8% против 40,0%) и BF-положительной (64,4% против 30,0%) по сравнению со штаммами, не относящимися к РеА (P <0,01).

Эти результаты позволяют предположить, что необходимо оценить функциональное значение продукции SIL и образования биопленок для изучения роли кишечных бактерий *Candida* в патогенезе спондилоартрита, включая реактивный артрит. Крайне важно определить микробные факторы, способствующие развитию РеА.

Ключевые слова: *Candida albicans*, фенотипические свойства, реактивный артрит

Introduction

At this time, reactive arthritis (ReA) is refers to an infection gastrointestinal tract induced systemic illness, characterized by a sterile synovitis occurring in a genetically predisposed individual [1, 2]. Numerous experimental studies have shown that the gut microbiota is the important pathogenetic factor in the development of spondylarthritis, including reactive arthritis. The connection of arthritis with dysbiotic gut microbiota disorders has been proved [3, 4]. A number of authors point to the role of fungi of the gut microbiota, and their phenotypic properties in the development of inflammatory diseases of the intestinal and extra-intestinal localization [5, 6]. It has been ReA subjects suffer from an intestinal dysbiosis characterized by an abnormal expansion of the *Candida* population [7]. *Candida albicans* is a gut commensal and opportunistic pathogen. Among virulence factors of *C. albicans* are of extracellular hydrolytic enzymes (lipases, phospholipases and secreted aspartyl proteinases), adherence and pleomorphism [8]. Research results demonstrate that the biofilm production could enhance the invasiveness and virulence of strains *C. albicans* [9]. In addition, a study by Deckers et al. [10] showed importance of the lysozyme inhibitors promote microorganisms in growth or survival in the ecological niches in the host. The results of others authors [11, 12] suggest that the inactivation of antimicrobial proteins (lysozyme, lactoferrin, defensins) may be important for commensal and pathogens to induce chronic inflammatory diseases of the human body. Thus, the aim of this work was to compare phenotypic properties of *Candida* species associated with the source of ReA, such as secretory inhibitor of lysozyme (SIL) and biofilm formation (BF).

Methods

This study included 109 children ranging in age from 5 to 16 years. Not all specimens yielded some fungi growth. A total of 65 non-duplicate, clinical isolates of Candida species, isolated from the feces of healthy children (n=20) and children with ReA (n=45). Forming of the investigated groups of children was conducted on the base of child's hospital № 6, Orenburg. On admission to the hospital the legal representatives of patient (mother, father, guardian) were acquainted and signed in a document about the informed voluntarily consent to medical interference that included a voluntarily consent to realization of necessary clinical and laboratory methods of research, including bacteriological and immunological methods (head 4 Federal laws from 21.11.2011 г. № 323-FL "About bases of health of citizens care in Russian Federation"). For the diagnosis of ReA, the criteria, approved by the International RA Meeting (Berlin,1996, 1999) was used.

Stool samples from children were homogenized in sterile 0.9% normal saline and cultured on Sabouraud glucose agar (2% D-glucose), incubated aerobically at 26 °C for 3–5 days and the values of log10 cfu/g were calculated. All fungal isolates grown on the selective medium were isolated to obtain single-cell pure colonies. Identification of Candida spp was with the use of the test system API20CAUX (bioMerieux, France). For the fungi to verify identification MALDI-TOF mass-spectrometr "Microflex" ("Bruker Daltonics", Germany) was used.

The strains were assess to SIL production used photometric method Bukharin O. V. et al. (1999). The biomass of the cultures to be tested for production of SIL was seeded with a standard bacteriological loop in 3 ml of liquid nutrient medium (Luria Bertani Broth, HiMedia) containing standard lysozyme solution (20 μg/mL) (Sigma Chemical Co., St Louis, MO, USA). After incubation for 24 h at 26 °C, then the optical density (OD) of broth culture against broth (Y) was measured on the elx808 photometer (BioTek, USA), wavelength 450 nm. The supernatant was separated from microbial cells by centrifugation at 3000 rpm for 15 minutes. As a test strain to determine of SIL used acetonized culture of *Micrococcus luteus ATCC 15307*. The culture was dissolved in a physiological solution, the optical density (OD) of the test strain was adjusted to 0.30 (0.28–0.32) on the elx808 spectrophotometer (BioTek, USA), wavelength 450 nm. Then 50 μl of the supernatant-lysozyme mixture was placed in

a polystyrene plate in vertical rows and the wells of each row of the tablet were filled with 200 µl suspension of Micrococcus by multichannel pipette. SIL production of each strain was indicated as according to the degree of lysis of the suspension and was expressed in µg/mL of inactivated lysozyme (mcg/ml*OD). SIL production of strain up than 0,50 mcg/ml*OD corresponded was SIL-positive strains.

Quantitative assessment of biofilm formation of *Candida* was performed using crystal violet binding assay method [13]. Briefly, strains were grown in Luria-Bertani broth for 48 h in ELISA plate together with negative control (the nutrient broth); growth was verified by measuring optical density (OD) with elx808 spectrophotometer. The number of inoculated plankton cells was calculated on elx808 spectrophotometer (BioTek, USA), wavelength 540 nm and expressed in conventional optical density units (OD). The analyses were performed in triplicates and the median value was used for analysis. After 48 hours, plankton cells of microorganisms were removed from the holes of the panel and biofilms were stained. To do this, 150 µl of distilled water and 20 µl of 1% crystal violet were gently (without stirring) introduced into the hole and incubated for 45 min at room temperature. After a thorough three-time washing with distilled water into the wells for the extraction of dye from the films were added 200 mcl of 96% ethanol and measured the optical density of this solution at a wavelength of 630 nm. The intensity of staining the contents of the holes corresponded to the quantity of BF. The ability to BF was expressed in units. Degree of biofilm formation was presented in conditional units (CU) which was the optical density of the broth after growth of the strain relative to the nutrient broth optical density (negative control). BF production of strain up than 1,0 CU corresponded was BF-positive strains.

The data are presented as the mean ± SD. The Wilcoxon signed-rank test was used to assess the statistical significance of difference between control and the samples. A value of $p < 0.05$ was considered statistically significant.

Results

Candida species were isolated more frequently from ReA group (93,8±6,5%) than from control group (32,8±2,6%) while *C. lusitanie, C. tropicalis* and *C. parapsilosis* were found only in specimens obtained from childrens with arthritis. The isolation of *C. albicans* and *C. krusei* was characteristic of both groups. In healthy children yeast were represented by the species *C. albicans* (75,0 ± 3,3%) and *C. krusei* (25,0 ± 1,1%). Among the *Candida* species in children with ReA, the species *C. albicans* dominated, which accounted for 55,6±3,7% of the total number of cultures. Other species included *C. krusei* – 17,8+1,2%; *C. lusitanie* – 11,1±0,7%; *C. tropicalis* 11,1±0,7% and *C. parapsilosis* – 4,4±0,3%. The concentration of microorganisms from control group were 3,1±0,3 $\log_{10}$ cfu/g versus 6,3±0.5 $\log_{10}$ cfu/g from ReA group.

Of the ReA strains tested, 35/45 (77.8%) were considered to be SIL-positive (*table 1*) compared with only 8/20 of the non-ReA isolates (40.0%, P< 0.05). Furthermore, a significantly higher proportion of ReA strains (64.0% vs. 30.0%) were BF-positive compared with non-ReA strains (P<0.01).

Table 1.
SIL and BF production of clinical isolates of Candida species
Abbreviations:
SIL, secretory inhibitor of lysozyme; BF, biofilms formation; ReA, chronic reactive arthritis

Organism	SIL-producing strains/total		BF-producing strains/total	
	Healthy childrens	ReA	Healthy childrens	ReA
C. albicans	7/15	21/25	5/15	19/25
C. krusei	1/5	5/8	1/5	4/8
C. lusitanie	0/0	4/5	0/0	3/5
C. tropicalis	0/0	3/5	0/0	2/5
C. parapsilosis	0/0	2/2	0/0	1/2

No. of organisms (healthy childrens / ReA)	No. of SIL-producing strains (healthy childrens /ReA) with different levels (µg mL-1 of inactivated lysozyme) of SIL			
	0–0,50	0,51–1,0	1,1–1,5	1,6–2,2
C. albicans (15/25)	8/4	7/6	0/13	0/2
C. krusei (5/8)	4/3	1/4	0/1	0/0
C. lusitanie (0/5)	0/1	0/3	0/1	0/0
C. tropicalis (0/5)	0/2	0/3	0/0	0/0
C. parapsilosis (0/2)	0/0	0/2	0/0	0/0

Table 2.
SIL production of clinical isolates of Candida species.
Abbreviations:
SIL, secretory inhibitor of lysozyme; ReA, chronic reactive arthritis

In contrast to the fungi isolated from the control group (*table 2*), strains isolated from childrens with ReA showed more intensive inhibition of the fungicidal activity of lysozyme (1,35±0,1 mcg/ml*OD vs. 0,71 ± 0,05 mcg/ml*OD, P < 0.05).

Among the BF-producing strains, fungi isolated from patients without ReA had mean BF production levels of 1,5±0,02 CU. The cultures of *Candida* from the ReA group were more active in biofilms formation (4,8±0,9 CU, P < 0.05). *C. albicans* strains ReA group were more potent biofilm producers than strains non-ReA group (7,7±0,5 CU vs. 1,5 ± 0,05 CU, P=0.048). In comparison with this, other types of yeast isolates from the ReA group showed a low activity of formation biofilm (*C. krusei* – 4,5±1,2 CU; *C. lusitanie* – 3,1±0,7 CU; *C. tropicalis* 2,1±0,7 CU and *C. parapsilosis* – 3,4±0,3CU, respectively).

Discussion

Interaction between an arthritogenic agent and a predisposed host is the basis for development of ReA [1]. Currently, changes in the intestinal flora and local changes in the balance between of pro- and anti-inflammatory factors of immunity are considered as a trigger for reactive arthritis [2, 3]. Opportunistic bacteria and fungi counteract the host immune defense by excreting various inhibitors. The inactivation of components of innate immunity (lysozyme) may be important for opportunistic pathogens to by avoiding clearance by microbicidal proteins and persistent surviving [5, 11, 12]. Biofilm is an important virulence factor *Candida* and also persistent surviving. Biofilm formation provides Candida species with the ability to evade host immune defenses and resistance to antifungals [14].

In this work, we showed that Candida strains from the feces in the ReA group have high values of biofilm formation and lysozyme inhibition (anti-lysozyme potential). These properties can be important in the survival of fungi and therefore can be influenced to prevent dysbiosis. Previously shown that gut dysbiosis (Candida yeast overgrowth) may be triggers of arthritis. It is obvious that SIL and BF also are markers of persistence of Candida, which colonizes the intestine in patients with RA. In this regard, we assume that the influence (reduction) of biofilm formation and inhibition of lysozyme can help achieve positive results in the prevention and treatment of Re A.

FUNDING:
This work was carried out in the framework of fundamental research at the Ural Branch of the Russian Academy of Sciences (project 18–7–8–34).

CONFLICTS OF INTEREST/COMPETING INTERESTS:
The authors declare that they have no competing interests.

AVAILABILITY OF DATA AND MATERIAL:
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

AUTHOR'S CONTRIBUTIONS:
Oleg Bukharin: Conceptualization, Methodology.
Natalya Perunova: Data curation, Writing- Original draft preparation.
Elena Ivanova: Visualization, Investigation.
Olga Chelpachenko: Writing – Reviewing and Editing.
Anatoly Khavkin: Writing – Reviewing and Editing

CONFLICTS OF INTEREST/COMPETING INTERESTS:
All authors listed on the title page are aware of this submission and all contributions are attributed in the author list or acknowledgment section.

ETHICS APPROVAL:
On admission to the hospital, the legal representatives (mother, father and guardian) of the patient were acquainted and signed a document about informed voluntary consent to medical interference, which included a voluntary consent to the realization of necessary clinical and laboratory research methods, including bacteriological and immunological methods (Head 4 of Federal Law from 21.11.2011 No. 323-FL "About bases of health of citizens care in Russian Federation").

CONSENT TO PARTICIPATE:
On admission to the hospital, the legal representatives (mother, father and guardian) of the patient were acquainted and signed a document about informed voluntary consent to participate for research.

CONSENT FOR PUBLICATION:
All authors read and approved the final manuscript.

Anatoly I. Khavkin, MD, PhD, DSc, Professor, Head of the Moscow Regional Center of Pediatric Gastroenterology, Hepatology; Chief Researcher of the Department of Gastroenterology, Veltischev Research and Clinical Institute for Pediatrics and Pediatric Surgery; Professor, Department of Pediatrics with a Course in Pediatric Surgical Diseases, Institute of Medicine; *ORCID: 0000–0001–7308–7280*

Natalia B. Perunova, Dr. Med. Sci., Professor of RAS, deputy Director for Research, Leading Researcher, Laboratory of Infectious Symbiology; *ORCID: 0000–0002–6352–8879*
Oleg V. Bukharin, Dr. Med. Sci., Academician of the RAS, Chief Researcher at the Institute of Cellular and Intracellular Symbiosis; *ORCID: 0000–0002–6039–5265*
E. V. Ivanova, Dr. Med. Sci., docent, leading researcher (with the duties of the head of the laboratory) Laboratory of Infectious Symbiology; *ORCID: 0000–0002–4974–8947*
Olga E. Chelpachenko, Dr Med Sci, Professor, Leading Researcher of the Laboratory of Biomonitoring and Molecular Genetic Research; *ORCID: 0000–0002–6719–5805*

Хавкин Анатолий Ильич, доктор медицинских наук, профессор, руководитель Московского областного центра детской гастроэнтерологии, гепатологии; главный научный сотрудник отдела педиатрии; главный научный сотрудник Научно-исследовательского клинического института педиатрии и детской хирургии имени академика Ю. Е. Вельтищева; профессор кафедры педиатрии с курсом детских хирургических болезней Медицинского института
Перунова Наталья Борисовна, д.м.н., профессор РАН, зам. директора по научной работе, ведущий научный сотрудник лаборатории инфекционной симбиологии
Бухарин Олег Валерьевич, д.м.н., академик РАН, Главный научный сотрудник

Иванова Елена Валерьевна, д.м.н., доцент, ведущий научный сотрудник (с исполнением обязанностей заведующего лабораторией) лаборатории инфекционной симбиологии
Челпаченко Ольга Ефимовна, д.м.н., профессор, ведущий научный сотрудник лаборатории биомониторинга и молекулярно-генетических исследований

Referenses

1. Stavropoulos P.G., Soura E., Kanelleas A., Katsambas A., Antoniou C. Reactive arthritis. *J Eur Acad Dermatol Venereol*. 2015 Mar;29(3):415–24. doi: 10.1111/jdv.12741.
2. García-Kutzbach A., Chacón-Súchite J., García-Ferrer H., Iraheta I. Reactive arthritis: update 2018. *Clin Rheumatol*. 2018 Apr;37(4):869–874. doi: 10.1007/s10067–018–4022–5.
3. Yeoh N., Burton J. P., Suppiah P., Reid G., Stebbings S. The role of the microbiome in rheumatic diseases. *Curr Rheumatol Rep*. 2013 Mar;15(3):314. doi: 10.1007/s11926–012–0314-y.

4. Sekirov I., Russell S. L., Antunes L. C., Finlay B. B. Gut microbiota in health and disease. *Physiol Rev*. 2010 Jul;90(3):859–904. doi: 10.1152/physrev.00045.2009.
5. Bukharin O.V., Perunova N. B. Microsymbiocenosis. Ekaterinburg. UrDepart RAS. 2014. (in Russ.)
6. Sonoyama K., Miki A., Sugita R., Goto H., Nakata M., Yamaguchi N. Gut colonization by Candida albicans aggravates inflammation in the gut and extra-gut tissues in mice. *Med Mycol*. 2011 Apr;49(3):237–47. doi: 10.3109/13693786.2010.511284.
7. Chelpachenko OE, Bukharin OV, Danilova EI, Fedotova LP, Perunova NB, et al. A clinical case of Candida arthritis in a child. *Vopr. prakt. pediatr. (Clinical Practice in Pediatrics)*. 2017;(3):77–82. (in Russ.) doi:10.20953/1817–7646–2017–3–77–82.
8. Höfs S., Mogavero S., Hube B. Interaction of Candida albicans with host cells: virulence factors, host defense, escape strategies, and the microbiota. *J Microbiol*. 2016 Mar;54(3):149–69. doi: 10.1007/s12275–016–5514–0.
9. Borghi E., Romagnoli S., Fuchs B. B., Cirasola D., Perdoni F., Tosi D., Braidotti P., Bulfamante G., Morace G., Mylonakis E. Correlation between Candida albicans biofilm formation and invasion of the invertebrate host Galleria mellonella. *Future Microbiol*. 2014;9(2):163–73. doi: 10.2217/fmb.13.159.
10. Deckers D., Vanlint D., Callewaert L., Aertsen A., Michiels C. W. Role of the lysozyme inhibitor Ivy in growth or survival of Escherichia coli and Pseudomonas aeruginosa bacteria in hen egg white and in human saliva and breast milk. *Appl Environ Microbiol*. 2008 Jul;74(14):4434–9. doi: 10.1128/AEM.00589–08.
11. Bukharin O. V. Persistence of pathogenic bacteria. Moscow. Medicine Publ., 1999. (in Russ.)
12. Ivanov I.B., Gritsenko V. A., Kuzmin M. D. Phenotypic differences between coryneform bacteria isolated from seminal fluid of healthy men and men with chronic prostatitis syndrome. *Asian J Androl*. 2009 Jul;11(4):517–20. doi: 10.1038/aja.2009.24.
13. O'Toole G.A., Kolter R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol*. 1998 May;28(3):449–61. doi: 10.1046/j.1365–2958.1998.00797.x.
14. da Silva Dantas A., Lee K. K., Raziunaite I., Schaefer K., Wagener J., Yadav B., Gow N. A. Cell biology of Candida albicans-host interactions. *Curr Opin Microbiol*. 2016 Dec;34:111–118. doi: 10.1016/j.mib.2016.08.006.