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Search for bacterial biofilms in the chronic anal fissure (descriptive study with in-depth visualization)

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ABSTRACT *AIM: to reveal biofilms in chronic anal fissure (CAF) bacterial composition.*
PATIENTS AND METHODS: patient A., 40 years old, with chronic posterior and anterior anal fissures with sphincter spasm underwent a lateral subcutaneous sphincterotomy with excision of the CAFs. Before the operation swabs were taken from fissures for microbiology. Removed specimens were placed in fixing solutions. Microbiological studies including 16S rRNA gene sequencing and MALDI-ToF mass spectrometry of isolated cultures were carried out to assess the bacterial composition of CAFs. Microscopic studies which included scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to search for the biofilm location of microorganisms. Conclusions about the presence of biofilms were made during the comparison of photographs with reliable images described in the literature.
RESULTS: a wide variety of bacteria (56 genera) was detected in the tissues forming the CAF based on sequencing of 16S rRNA genes; swab's mass spectrometry revealed only E. coli and P. anaerobius in significant concentrations. Using SEM on one of the sections of the excised CAF were found biofilms containing bacterial cells immersed in an extracellular matrix (which size was within the limits of 2 microns); similar structures weren't detected outside the CAF. On TEM an accumulation of bacterial cells, surrounded by an extracellular polymer matrix, what was interpreted as a biofilm of gram-negative bacteria, was identified. After 7 months, the patient retained a non-healing postoperative wound in the area of the previously excised posterior fissure. After repeated sowing, E. coli and S. gallolyticus were obtained in significant concentrations. Excision of the scarred edges of the wound and the prescribed conservative treatment allowed the wound to epithelize within 3 weeks.
CONCLUSION: the results show that microbial biofilms can be localized in the CAF. Further researches are needed for reliable conclusions about biofilm organization in CAF and their effect on pathological and reparative processes.

KEYWORDS: *chronic anal fissure, CAF, biofilm, lateral internal sphincterotomy, scanning electron microscopy, SEM, transmission electron microscopy, TEM, 16S rRNA sequencing, MALDI-ToF mass spectrometry*

CONFLICT OF INTEREST: *the authors declare no conflict of interest*

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INTRODUCTION

A chronic anal fissure (CAF) is an anodermic ulcerative defect localized in the area of the 'anatomical' anal canal that exists for more than 2 months [1], and therefore CAF can be regarded as a long-term non-healing wound.

Long-term non-healing wounds contain a wide variety of microorganisms, including pathogenic ones, whose virulence factors and waste products lead to a prolonged wound condition in the inflammatory phase, which prevents epithelialization and healing of the wound defect [2]. Under certain conditions, these microorganisms have the ability to form biofilms. According to a systematic review and meta-analysis conducted by Malone, M. et al. (2017), the prevalence of biofilms in long-term non-healing wounds of various etiologies

(diabetic foot ulcers, pressure sores, non-healing surgical and unspecified chronic wounds) averages 78%, ranging from 60% to 100% [3].

In a study by Kozlovska et al. (2018) of the microflora of over 100 CAFs, none of the antimicrobial drugs demonstrated one hundred percent bactericidal action. The authors attribute the resistance of microorganisms to the fact that the bacteria were protected by a high-density biofilm matrix [4]. However, they used only cultural diagnostic methods without visualization of microbial biofilms. Our hypothesis is that microbial biofilms, along with planktonic microorganisms, can be localized in CAF and interfere with the healing of a wound lesion.

PATIENTS AND METHODS

Terminology

By biofilms, we mean a structured microbial consortium enclosed in an exopolymer matrix that protects its constituent organisms from the host's immune response and reduces sensitivity to drugs aimed at the eradication of microbes from wounds [5–7].

Study Design

In order to search for biofilms in CAF and evaluate the spectrum of bacteria in it, the following study design was formulated (Fig. 1). The inclusion criterion is a patient with CAF with confirmed spasm of the sphincter without concomitant pathology. Before the surgery, swabs are taken from the fissure for further cultural microbiological studies, after which the surgery is performed in the volume of a lateral subcutaneous sphincterotomy with excision of the fissure. Next, the excised tissues forming the CAF are prepared for further study. Scanning electron and transmission electron microscopy are used to search for microbial biofilms. 16SrRNA sequencing and MALDI-ToF mass spectrometry are used to evaluate the CAF microbiome.

Characteristics of Clinical Observation

Patient A., 40 years old, turned to the polyclinic of the RNMRC of Coloproctology of the Health Ministry of Russia, complaining of pain in the anus during and after defecation, periodic discharge of blood. These symptoms bothered the patient for a year; conservative treatment methods had no effect. When examining the anal canal at 6 and 12 o'clock on conventional dial, chronic anodermic defects were visualized.

During the examination, the patient experienced severe pain. Based on anamnesis, objective examination and profilometry, the diagnosis of K.60.1 Chronic posterior and anterior anal fissures with spasm of the sphincter was established. The patient was hospitalized at the Center, and a surgery

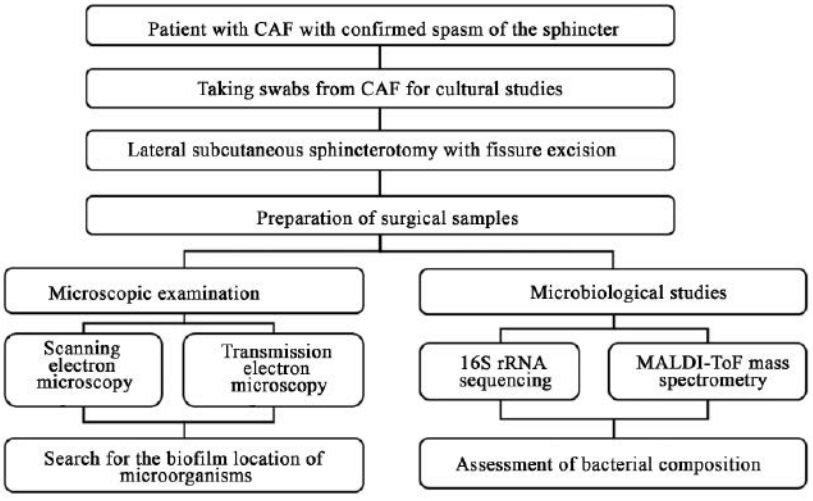

Figure 1. *Research design*

was performed in the volume of a lateral subcutaneous sphincterotomy with excision of 2 fissures.

Sample Preparation

Before the surgery, swabs were taken from 2 CAFs for further identification of aerobic and anaerobic bacteria localized in them. After excision of fissures, tissue samples were fixed on pins and placed in solutions for further transportation and storage. Samples of the anterior CAF were taken for transmission electron microscopy, and the posterior CAF — for 16SrRNA sequencing and scanning electron microscopy.

MALDI-ToF mass spectrometry

Isolated colonies obtained during primary growth on dense nutrient media were used for species identification of isolated microorganisms. Bacterial cultures were subjected to preliminary sample preparation (extraction) and the method of direct deposition of the material on the mass spectrometer target was used. After applying the culture to 2 cells of the steel target, 1 mcl of 70% formic acid was added, dried in air, and 1 mcl of a matrix consisting of α-cyano-4-hydroxycinnamic acid (α-CHCA) and 50.0% acetonitrile / 2.5% trifluoroacetic acid (NPF Litech LLC) was applied on top. After drying of the matrix, the target was placed in a Microflextime-of-flight mass spectrometer (Bruker Daltonics, Germany). External calibration was performed using accurate mass values of well-characterized mixtures of lyophilized *E. coli* proteins. The identification of microorganisms and the calculation of the confidence coefficient (CC) were carried out automatically; for each result, a link was provided to the NCBI (National Center for Biotechnology Information). CC ⩾ 2.0 indicated identification with accuracy to the species; 1.7–2.0 – to the genus; < 1.7 – negative.

16SrRNA Sequencing

DNA was isolated using the DN easy Power Soil Kit (Qiagen, Germany), following the manufacturer's protocols. The variable V3-V4 region of the 16S rRNA gene was amplified using universal primers 341F CCTAYGGGDBGCWSCAG and 806R GGACTACNVGGGTHTCTAAT [8]. The obtained PCR fragments were used to prepare libraries for subsequent sequencing using the Nextera XT DNA Library Prep Kit (Illumina), following the manufacturer's protocols. Multiplexing was performed using the Nextera XT Index Kit v2 kits. PCR fragments were sequenced using Illumina MiSeq. The readings were combined, eliminating low-quality readings, singletons, and chimeras. Next, clusterization of the readings remaining after these procedures was performed in the OTU with a minimum identity of 97%. In order to determine the proportion of OTU in each of the samples, the original readings (including low-quality and singletons) were superimposed on representative OTU sequences with a minimum identity of 97% over the entire length of the reading. These procedures were performed using the use arch software package [9]. Based on the obtained 16SrRNA gene sequences, a taxonomic identification of microorganisms was performed using use arch and the Silva database.

Scanning Electron Microscopy (SEM)

For SEM, samples were fixed in 2.5% glutaraldehyde solution and washed twice with sodium phosphate buffer, after which they were sequentially treated with ethanol in increasing concentrations: 30% (for 1–2 min.), 50% (5–10 min.), 70% (5 min.) and 96% (5 min.). The samples were attached to columns for scanning microscopy (JEOL-IT 200 microscope, Tokyo, Japan) with double-sided adhesive tape and coated with gold plating.

Transmission Electron Microscopy (TEM)

The sample was immediately placed in a 2.5% glutaraldehyde solution in a cacodilate buffer (0.05 M sodium cacodilate solution, pH 7.0–7.5) and kept for a day at 4°C; then washed three times with the same buffer solution for 5 minutes and fixed in a solution of 1% OsO_4 in 0.05 M cacodilate buffer. After fixation in OsO_4, the samples were cut into pieces of approximately 1.5 by 1.5 mm. They were successively soaked in 3% uranyl acetate

Table 1. *Bacterial spectrum of microorganisms obtained as a result of MALDI-ToF mass spectrometry of swabs taken from the anterior and posterior CAF*

Name of the microorganism	Concentration (CFU/ml)
Anterior fissure	
Escherichia coli	10^4
Posterior fissure	
Escherichia coli	10^8
Peptostreptococcus anaerobius	10^7

solution and 30% ethyl alcohol for 2 hours, then in 70% ethanol for 12 hours at 4°C. The material was dehydrated in 96% ethyl alcohol (2 times for 10 minutes), then in absolute acetone (3 times for 10 minutes). The samples were poured into Epon 812 resin (Epoxy Embedding Medium Epon® 812, Sigma-Aldrich, USA) and soaked in a resin mixture: acetone in a 1:1 ratio for 2 hours, and then the mixture ratio was changed to 2:1 and kept for another 2 hours. The dehydrated material was placed in capsules with resin and kept at 37°C for 24 hours for impregnation, then at 60°C for 24 hours for polymerization. Ultrathin sections were obtained on an LKB-III microtome (LKB, Sweden), contrasted in a 3% uranyl acetate solution, then using Reynolds' method [10].

The obtained specimens were analyzed using a JEM100SHP electron microscope (JEOL, Japan).

The photo documentation of the materials was carried out using the MoradaG2 digital optical image output system.

The conclusion about the presence of biofilms was made based on a comparison of photographic images with reliable images of biofilms described in the literature [11–15].

Taxonomic Trees

In order to visualize the hierarchy of isolated microorganisms during 16SrRNA sequencing from genus to kingdom, taxonomic trees were constructed in RStudio (Rv. 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria)) using the yatahv. 1.0.0. library.

According to the list of identified genera of microbes, their complete classification was carried out from the NCBI database using the Rtaxize v.0.9.100 library. If automatic classification could not be performed, the search was carried out manually in NCBI or in the SeqCode register. If it was not possible to determine 1 of the hierarchy levels in this case, omissions were left.

RESULTS

MALDI-Tof Mass Spectrometry

MALDI-Tof mass spectrometry identified *E. coli* at a concentration of 10^4 CFU/ml from the swabs taken from the anterior fissure; *E.coli* was also identified from the posterior fissure, but at a higher concentration (10^8 CFU/ml), and *Peptostreptococcus anaerobius* (10^7 CFU/ml). The results are presented in Table 1.

16S rRNA Sequencing

Based on the results of reading the gene sequences obtained during 16SrRNA sequencing, the identification of bacteria was carried out. Some have been successfully classified to a genus, others to the level of a family, order, class, or just a type.

The most common types were Bacillota (47.0%), Pseudomonadota (18.0%), Bacteriodota (12.0%), the remaining types at the level of 12.2%, unclassified 10.7%. The predominant classes were Erysipelotrichia (37.9%), Gammaproteobacteria (16.9%), Bacteroidia (11.8%), and Bacilli (6.4%), with other classes accounting for 15.6%, and 11.4% without successful identification. At the order level, the predominant bacteria were Peptostreptococcales and Tissierellales (34.8%), Bacteroidales (11.1%), Pseudomonadales (9.7%), Lactobacillales (5.9%), other orders (26.3%), unclassified (12.2%). By families: Peptostreptococcaceae (33.9%), Prevotellaceae (9.0%), Pseudomonadota (8.1%), Streptococcaceae (5.9%), the rest (29.6%), 13.6% could not be classified. At the genus level: Peptostreptococcus

(11.2%), Pseudomonas (8.1%), Finegoldia (8.1%), Peptoniphilus (6.6%), Prevotella (6.1%); other genera accounted for 38%, unidentified 22%.

For 56 successfully identified bacteria, a taxonomic tree was constructed up to the genus level, shown in Fig 2.

In addition, two genera related to archaea have also been identified: Woesearchaeales and A-plasma.

It is worth noting that as a result of microbial profiling, all the bacteria contained in the biopsy were identified, some of which could not be associated with the pathological process.

SEM

When analyzing the images obtained with scanning electron microscopy (SEM), the areas both inside and outside the CAF were compared.

A uniform surface identified as anal sphincter tissue was visualized outside the fissure (Fig. 3a).

Objects similar to bacterial cells were not found.

A similar pattern was observed on most of the surface of the excised CAF, and only in one area were formations similar to biofilms characterized by the presence of individual bacterial cells up to 2 microns in size immersed in an extracellular polymer matrix, as judged by its mucous nature (Fig. 3b).

TEM

When examining photographs of excised CAF samples obtained by transmission electron microscopy, objects were also found that were morphologically identified as biofilms (Fig. 4–5).

A formation of cells (Fig. 4) measuring 0.2–0.6 microns was found inside the tissue, which corresponds to the size of small bacterial cells. The cells have a rounded shape and are gram-negative cells, because they consist only of an electron-dense cytoplasm surrounded by a two-layered cell wall 14–20 nm thick (Fig. 4c). The cells are surrounded by an extracellular polymer matrix with a clear boundary. The cytoplasm of cells is dense, the details of the structure are indistinguishable, which corresponds to some forms of rest [16]. Membrane vesicles characteristic of biofilms are also visualized between cells [17].

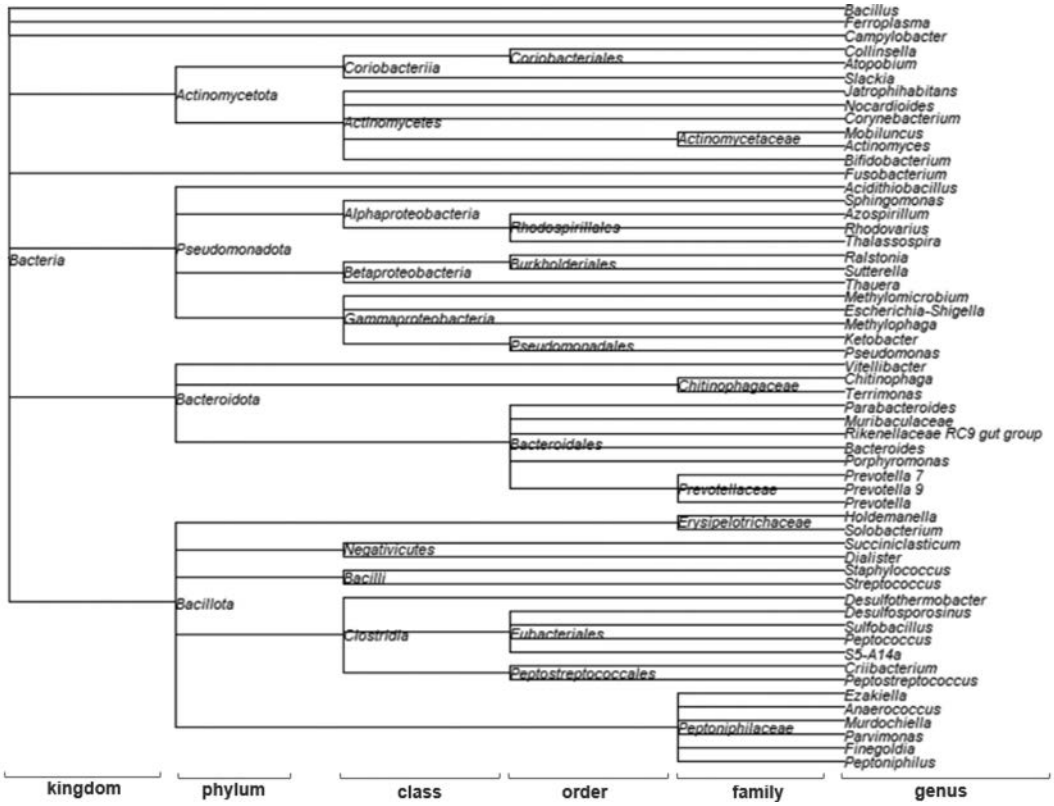

Figure 2. *A taxonomic tree drawn from identifiedbacterialgenusafter16SrRNAsequencing of a sample of excised anterior CAF*

Biofilms in the stage of decomposition (dispersion) were found in other parts of the CAF sample (Fig. 5 a,b,c). The surrounding tissue is also in a state of decomposition (possibly autolysis). The cytoplasmic membranes are preserved, and the intracellular contents are missing in whole or in part, the extracellular matrix is also fragmented, and the outer boundary of the biofilm is missing. The shape of the cells varies from rounded to elongated, the length ranges from 150 to 1,000 nm. The thickness and structure of the cytoplasmic membrane correspond to the bacterial one. Some cells have membrane structures.

Biofilms in the decomposition stage, consisting of whole and lysed L-forms, were found in some areas of the biopsy (Fig. 6).

In addition, collagen fibers were visualized in longitudinal (Fig. 7a) and transverse (Fig. 7b) sections in many photographs of excised CAF samples.

Treatment Results

Patient A. did not undergo follow-up examinations after the surgery and turned to the Center only 7 months after it. At 12 o'clock on conventional dial, the wound was epithelized, whereas at 6 o'clock on dial, the postoperative wound persisted and was regarded as long-term non-healing. Bacterial cultures were swabbed from its depth, which revealed *E. coli* at a concentration of 10^7 CFU/ml and *S. Gallolyticus* — at 10^8 CFU/ml (Table 2). The cicatricial edges of the wound were excised, and the patient was prescribed conservative treatment: ornidazole (an antiprotozoal drug with activity

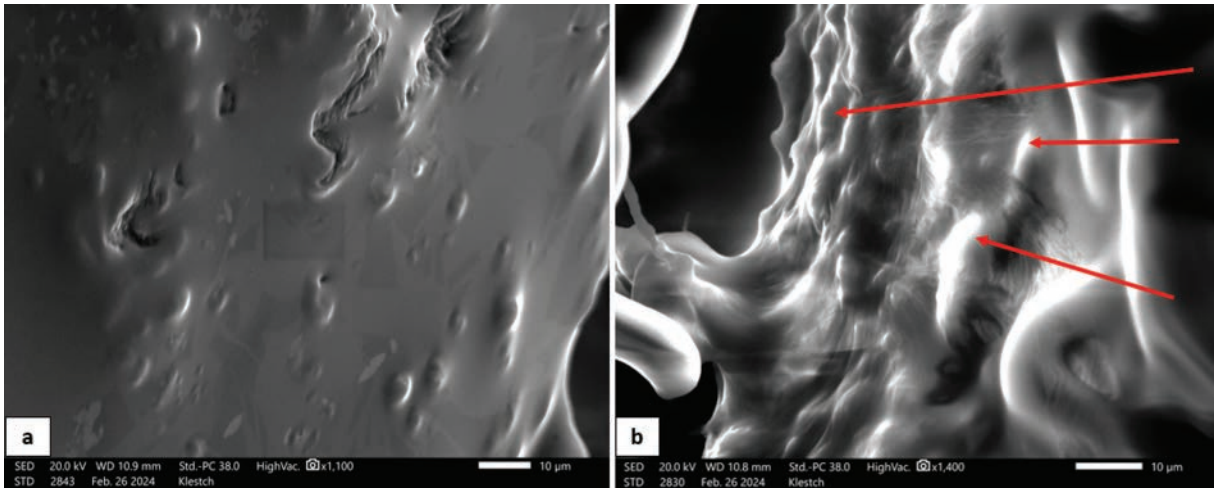

Figure 3. *Images obtained from SEM results (scale bar 10 mm). a) The surface of the internal anal sphincter tissue; b) the surface of the excised CAF. The arrows point on structures which were identified as biofilms*

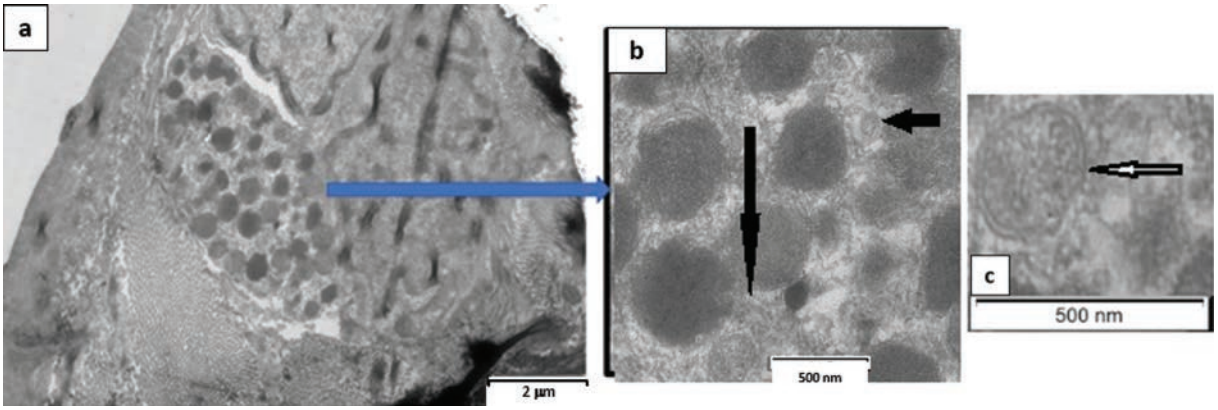

Figure 4. *The image obtained from the results of TEM. Microbial biofilm represented by a cluster of bacterial cells: a) general view of the biofilm (scale bar 2 mm); b) enlarged image a (scale bar 500 nm), membrane vesicles are shown by arrows; c) a double-layered cell wallis shown by an arrow (enlarged image a (scale bar 500 nm))*

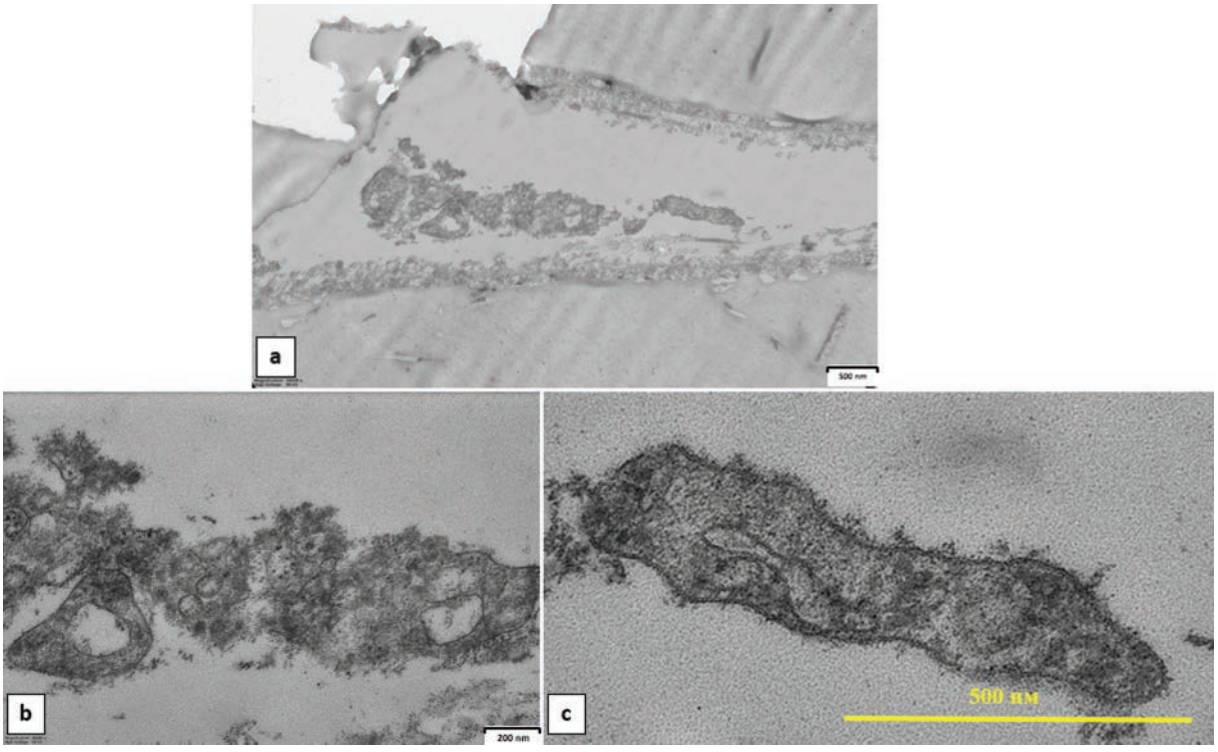

Figure 5. *The images obtained from the results of TEM. Biofilms in the decomposition stage. a) 500 nm scale bar; b) 200 nm scale bar; c) 500 nm scale bar*

against anaerobic cocci), rinsing the wound with a solution of Metrogil (active ingredient: metronidazole), applying Stellanin ointment (stimulates tissue regeneration) and Prontosan gel (helps to cleanse, moisten the wound and suppress bacterial flora). Epithelialization of the postoperative wound occurred 3 weeks after the start of the treatment. It is worth noting that no manometric signs of spasm of the internal anal sphincter were detected.

DISCUSSION

Currently, there are studies in which the bacterial picture of acute and chronic anal fissures has been studied, including the elimination of the microbial component from this zone through the use of antibacterial drugs [4,18–21]. This suggests that there are researchers in the world who consider the microbial factor as one of the causes of prolonged non-healing of the anal fissure and its transition to a chronic form.

Bacteria can exist both in the form of planktonic forms and in the form of organized microbial aggregations — biofilms. The current understanding of the existence of microorganisms in the form of biofilms on abiotic surfaces is adequately illustrated in the literature review by Sauer K. et al. (Fig. 8) [22]. However, the tissues of the human body are more labile, and recently there have been studies on biofilms on and in human tissues.

It is for such biofilms that participation in pathological processes is shown. They are found both in the intercellular space and inside human cells, which was summarized in the literature review by Mirzaei R. et al. (Table 3) [23].

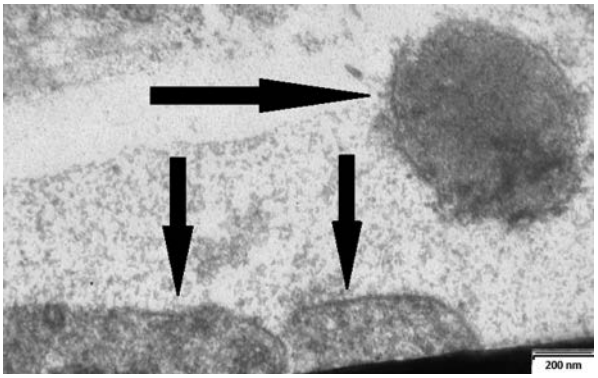

Figure 6. *The images obtained from the results of TEM. The arrows indicate L-forms (scale bar 200 nm)*

Table 2. *Bacterial spectrum of microorganisms obtained as a result of MALDI-ToF mass spectrometry of swabs taken from a long-term non-healing postoperative wound at 6 o'clock on dial*

Name of the microorganism	Concentration (CFU/ml)
Long-term non-healing postoperative wound at 6 o'clock on dial	
Escherichia coli	10^7
Streptococcus gallolyticus	10^8

Table 3. *Bacterial infections associated with intracellular biofilms in humans [23]*

Infectious disease	Organism
Urinary tract infection (UTI)	Uropathogenic *Escherichia coli* (UPEC), *Klebsiella pneumoniae, Enterococcus faecalis*
Lung infections (cystic fibrosis)	*Pseudomonas aeruginosa*
Otitis media	*Haemophilus influenzae and Streptococcus pneumoniae*
Alzheimer's disease	Spirochetal bacteria
Tonsillitis	Group A streptococci

The difference between intracellular and extracellular bacterial biofilms is particularly significant, since the former option provides a different level of protection from both antibacterial drugs and immune cells, which makes their therapy more difficult.

For our study, infections of the genitourinary tract associated with *E. coli* are of interest, since it is these microorganisms that were obtained by MALDI-Tof mass spectrometry, and we can assume that in our case the mechanisms of biofilm formation may be similar.

Since we were unable to find other studies on microscopy of biofilms of anal fissures, more studied biofilms of bladder stones [11,12], diabetic foot ulcers and chronic wounds of other etiology were used for comparative study [13–15].

According to Worlitzsch D. et al.'s study [24] pathogen cells and biofilms may be absent on the surface of the epithelium, but they can form in human tissue under epithelial cells.

Based on these considerations, we searched for microbial cells both on the tissue surface (SEM) and in the tissue thickness (TEM). According to the SEM results, cells were found on the fissure surface in only one location (Fig. 3b). This is consistent with the results, for example, on biofilms on the skin surface [25] wherein they are in small fragments and in small numbers. Despite the fact that samples for SEM are subjected to harsh drying treatment, an analysis of the literature shows that the formations found correspond to cells under the matrix layer (Fig. 4 –as per Holmberg A. et al.'s study [26] and Fig. 3 — as per Davis L.E. et al.'s study [27]).

More objects similar to bacterial cells were found on TEM. The most interesting finding is presumably a biofilm, the cells of which correspond to *E. Coli* (Fig. 4). A similar structure was shown in Cai Y.M. et al.'s study [28], in which biofilms of pathological pulmonary processes were studied.

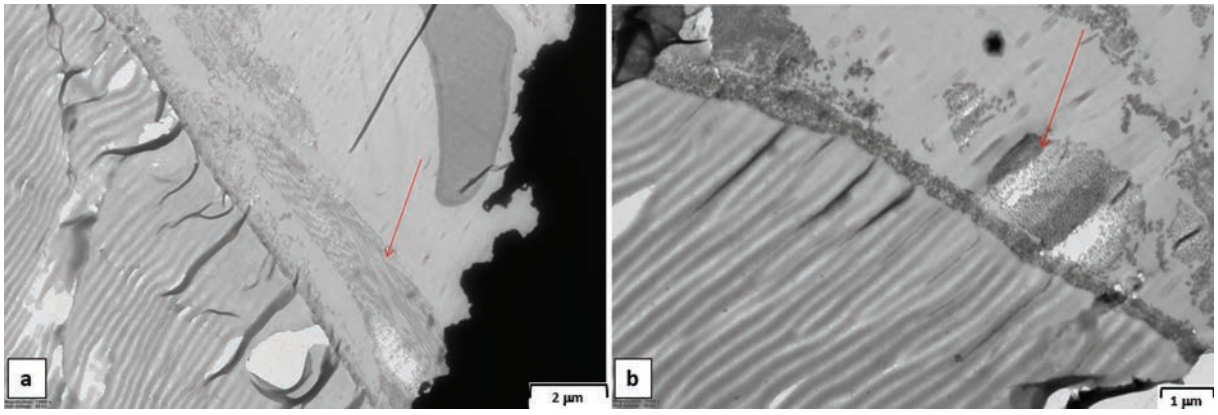

Figure 7. *The images obtained from the results of TEM. Collagen fibers in a) longitudinal (scale bar 2 mm) and b) cross (scale bar 1 mm) sections in the excised CAF. The arrows indicate collagen fibers*

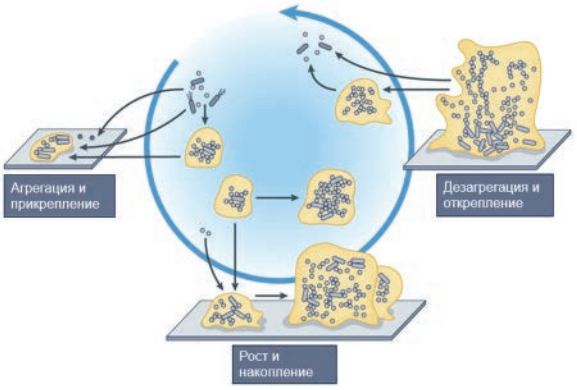

Figure 8. *A modern model of the existence of microorganisms in the forms of biofilms and plankton cultures [22]*

In addition, the TEM method revealed a large number of formations morphologically similar to the resting forms of microorganisms that the authors observed on other objects [29].

It is worth paying attention to the fact that the biofilm organization of microorganisms prevents the action of antibacterial substances, as shown by Kozlovska I. et al. based on CAF materials in the laboratory [4].

However, in a study by Grekova N.M. et al. the treatment of CAF with suppositories with 250 mg of metronidazole significantly reduced the healing time, reaching 61% epithelialization on day 10, compared with the control group without AB therapy (suppositories with 5 mg of hydrocortisone and 5 mg of cinchocaine hydrochloride for 14 days + suppositories with 250 mg of sodium alginate for another 14 days), amounting to only 12.5% [18]. Perhaps, in Grekova's study, the tactics of managing patients in the main group allowed the exopolymer matrix of the biofilm to be destroyed and the wound to exit the inflammatory phase, or the microbial factor really played a leading role in the pathogenesis of the fissure, but the bacteria were not organized into biofilms, which is why antibiotics were able to act so quickly and significantly accelerate the reparative processes.

In our case, surgical removal of the spasm of the internal anal sphincter allowed only the postoperative wound to epithelize at 12 o'clock on dial, whereas at 6 o'clock on dial in 7 months after the treatment, the wound persisted, and *E. coli* and *S. Gallolyticus* were seeded from it in significant concentrations. Subsequently, conservative therapy with antibacterial drugs promoted wound healing within 3 weeks. Therefore, the question of the role of biofilms in the pathogenesis of CAF and the possibility of their elimination by antibacterial drugs requires further study.

CONCLUSION

The results obtained indicate that microbial biofilms can be localized in the CAF. For reliable conclusions about the biofilm organization of microorganisms in CAF and their role in pathological and reparative processes, further research in this direction is needed. The study was carried out with the support of the Ministry of Science and Higher Education of the Russian Federation (state assignment No. 122040800164-6).

AUTHORS CONTRIBUTION

Concept and design of the study: *Maria A. Ignatenko, Aleksey A. Ponomarenko, Marina V. Zhurina, Yuri A. Nikolaev*

Collection and processing of the material: *Nadezhda A. Kostrikina, Marina V. Zhurina, Aleksey A. Ponomarenko, Evgeny E. Zharkov, Alina R. Melkumyan*

Text writing: *Maria A. Ignatenko*

Statistical processing: *Maria A. Ignatenko*

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