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NEW METHOD FOR DIAGNOSTICS OF INFECTIOUS AND INFLAMMATORY DISEASES OF THE MALE LOWER GENITAL TRACT

("Androflor®", "Androflor® Screen" kits)

STUDY GUIDE

Moscow, 2019

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New method for diagnostics of infectious and inflammatory disease of the male lower genital tract («Androflor®», «Androflor® Screen» kits).

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This guide is devoted to the diagnostics of the male lower genital tract infectious and inflammatory diseases. The guide presents the current concepts about male genital tract microbiota (taking into account large-scale studies of the last decade), modern techniques for studying this microbiota, and diagnosing infectious genital pathology. The innovative molecular-based technique for assessment of the male lower genital tract microbiota is presented.

CONTENTS

ABBREVIATIONS

BV — bacterial vaginosis
CT — Chlamydia trachomatis
DNA — deoxyribonucleic acid
IVF — in vitro fertilization
FVU — first void urine
GE — genomic equivalents
HGD — human genomic DNA
HPV — Human Papilloma Virus
HSV — Herpes Simplex Virus
IUSTI — International Union against Sexually Transmitted Infections
LUTS — lower urinary tract symptoms
MG — Mycoplasma genitalium
NAAT — nucleic acid amplification test
NG — Neisseria gonorrhoeae
NGU — non-gonococcal urethritis
NCNGU — non-chlamydial non-gonococcal urethritis
OM — opportunistic microorganisms
PCR — polymerase chain reaction
GP — glans penis
PMNL — polymorphonuclear leukocytes
FS — foreskin
ROC — receiver operating characteristic
RT-PCR — real-time polymerase chain reaction
STI — sexually transmitted infection
SW — software
TBL — total bacterial load
TV — Trichomonas vaginalis
UV — ultraviolet

I. INTRODUCTION

Infectious and inflammatory diseases of the male urogenital system are among the main causes of reproductive disorders, infertility and reduced quality of life which is of great socio-economic importance.

The tissue damage occurring during infectious and inflammatory process could be caused by direct impact of microbial virulence factors. The inadequate inflammatory reaction to pathogenic microorganisms or high quantities of opportunistic microorganisms (OM) enhances the pathological process indirectly.

The severity of reproductive tract tissue damage depends on the duration and the intensity of inflammation. Chronic inflammation has a more prolonged toxic effect on the tissue and can also lead to the development of autoimmune diseases in individuals with genetic predisposition to adverse immune reactions.

Diseases of the lower urogenital system are widespread in men, especially sexually active ones. Balanitis or balanoposthitis make up to 11% of male urogenital diseases and can be recurrent or persistent [1]. Balanitis is caused by an infection in approximately 60% of cases [2]. Balanitis / balanoposthitis can be caused by *Candida spp*., aerobic and anaerobic bacteria, *Gardnerella vaginalis*, often in combination with anaerobic bacteria, viruses, parasites and other agents of sexually transmitted infections (STIs). Non-infectious pathology could be associated with mechanical stress [2], lichen planus, psoriasis and contact dermatitis [3].

Urethritis is the most common urogenital tract disease in men. Although non-infectious causes are known, most cases of urethritis are associated with infections [4]. The most common causes of urethritis are *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT). When NG and CT are absent the term "nonspecific non-chlamydial non-gonococcal urethritis" (NCNGU) is used. The development of NCNGU is associated with *Mycoplasma genitalium* (MG), *Trichomonas vaginalis* (TV) and much less often with herpes simplex virus (HSV) or adenovirus [5]. In some studies, *Ureaplasma urealyticum* was given the leading role in the development of urethritis symptoms [6–8]; however other studies present different data [8, 9]. No pathogen was detected in almost 50% of cases of NCNGU [5]. NCNGU in men could be associated with bacteria that cause diseases of female reproductive tract [5, 6], as well as other groups of difficult to culture or non-culturable obligate anaerobic bacteria [8-10]. That is why their role in the development of inflammatory diseases of the male urogenital system is uncertain [8–10].

Currently, culture-independent methods, such as real-time polymerase chain reaction (RT-PCR), which allow quantitative assessment of microbiota including difficult to culture and non-culturable obligate anaerobic microorganisms, are perhaps the only alternative to standard laboratory techniques.

II. INDICATIONS AND CONTRAINDICATIONS FOR USING THE METHOD

Indications:

- the presence of complaints and / or clinical symptoms of inflammation of the lower urogenital tract in men: discharge from the urethra, urination disorders, discomfort in the urethra, redness, itching, swelling, rash on the head of the penis, unpleasant smell, etc.;
- reproductive function disorders, infertility, preparation for IVF;
- assessment of the effectiveness of therapy and treatment outcomes.

There are no contraindications.

III. MATERIALS AND TECHNICAL SUPPORT OF THE METHOD

- PCR hood with a UV lamp.
- Real time PCR thermocycler: “DTprime” (“DNA-Technology”, LLC), “DTlite” (“DNA-Technology”, LLC).
- Software and ini-file with “Androflor®” analysis parameters.
- Household refrigerator with freezer.
- Mini-centrifuge/vortex.
- 0.2 ml tube rack.
- Variable volume pipette with disposable tips (2.0-20 µl, 10-100 µl).
- Pipette tips with a capacity of 1.0-200 µl, 100-1000 µl.
- Container with disinfectant solution for disposing used tips, test tubes and other consumables.
- The kit for nucleic acids isolation PREP-NA/PREP-NA-PLUS (“DNA-Technology”, LLC).
- Kit for analyzing microbiocenosis of the male urogenital tract by means of RT-PCR: Androflor® and Androflor® Screen (“DNA-Technology”, LLC).

IV. DESCRIPTION OF THE METHOD

Androflor® and Androflor® Screen determine the etiology of infectious and inflammatory processes in the lower urogenital tract of men with the following nosologies (ICD10):

N34.1 Nonspecific urethritis
N34.2 Other urethritis
N34.3 Urethral syndrome, unspecified
N40 Enlarged prostate
N41.0 Acute prostatitis
N41.1 Chronic prostatitis
N45 Orchitis and epididymitis
N48.1 Balanitis
N48.6 Induration penis plastica
N49.0 Inflammatory disorders of seminal vesicle
N49.1 Inflammatory disorders of spermatic cord, tunica vaginalis and vas deferens

Both obligate sexually transmitted pathogens and opportunistic microorganisms (the yeast-like fungi of the *Candida spp*., protozoa, and others) can be the cause of these diseases.

A number of laboratory methods are recommended for diagnosis of these conditions. According to International Union against Sexually Transmitted Infections (IUSTI) the diagnosis of urethritis is confirmed by demonstrating an excess of polymorphonuclear leukocytes (PMNLs) in the anterior urethra. Gram-stained or methylene-blue stained urethral smear is used for this purpose. In absence of *N. gonorrhoeae,* testing for *C. trachomatis*, *M. genitalium* and *T. vaginalis* by nucleic acid amplification test (NAAT) is highly recommended. In men at low risk for a sexually transmitted infection, a urinary culture should be considered [11].

Balanitis describes inflammation of the glans penis, posthitis inflammation of the foreskin. In case the infectious origin is suggested sub-preputial swab for *Candida spp.* and bacterial culture should be undertaken according to IUSTI guidelines. Testing for *C. trachomatis* and *T. vaginalis* by NAAT should be considered when female partner has an undiagnosed vaginal discharge [12].

Bacterial prostatitis, with confirmed or suspected infection, must be distinguished from chronic pelvic pain syndrome. The European Association of Urology recommends a number of microbiology tests for this purpose. The most important test in the evaluation of a patient with acute and chronic bacterial prostatitis is urine culture. The four-glass

Meares and Stamey test is the optimum for diagnosis of chronic bacterial prostatitis. First-void urine is the preferred specimen for the diagnosis of urogenital *C. trachomatis* infection in men by NAATs. Semen culture sensitivity is reported to be approximately 50%; therefore, it is not routinely part of the diagnostic assessment of chronic bacterial prostatitis [13].

Until recently, all data on possible infectious pathogens of the male urogenital tract were obtained using culture-based technique — which to date continues to be the gold standard for diagnosis of infectious and inflammatory processes. However, in recent years, molecular techniques have been introduced into practical medicine. The spectrum of the etiologically significant microorganisms has been significantly expanded due to the discovery of new difficult to culture or non-culturable microorganisms that were previously unknown.

According to modern views, the diseases of the male urogenital tract could be caused either by obligate pathogens (*Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium, Trichomonas vaginalis*), or by opportunistic microorganisms which normally can be present in the urinary tract in small numbers. This is why quantitative evaluation is required for establishing their role in the development of urogenital diseases. Also commensals like *Staphylococci spp., Streptococci spp., Corynebacterium spp., Lactobacillus spp*. could be found in small numbers in lower urinary tract (distal urethra, glans penis, coronal sulcus) of asymptomatic men [14, 15].

The quantity of opportunistic microbiota can be established by means of culture-based and culture-independent molecular techniques. Despite a number of limitations and requirements for the research protocol, culture-based techniques are more commonly used in practical medicine.

Opportunistic pathogens are not only widely spread in the environment, but they can also contaminate a specimen during sampling, therefore, it is necessary to follow the rules for obtaining and transportation of clinical sample for testing. It is necessary to deliver the sample as quickly as possible to the laboratory in order to preserve the proportion between microorganisms which stability *in vitro* is different (like the need for oxygen prior to the inoculation of the culture media). It is also necessary to ensure equal conditions for the growth of different microorganisms (for example aerobic and anaerobic ones) to maintain the initial proportions between them.

The analysis time is also of great importance, so the doctor could make a decision regarding the appropriate course of treatment. Culture-based techniques are quite demanding, as well as time-consuming (7–10 days), so the treatment is often empirical at the start of therapy. It could involve excessive prescription of drugs and, accordingly, an increased risk of adverse effects.

In recent years, culture-independent molecular-techniques based on microbial nucleic acid analysis have been developing rapidly. One of them is the polymerase chain reaction method with real-time recording of results (RT-PCR). This method enables quantitative analysis of any number of microorganisms in a biological sample.

The method was developed in 1996 [16] following the "classic" PCR. The real-time PCR method provides high sensitivity and equivalent specificity of the "classic" PCR, while at the same time it has several advantages. Since amplification and detection occur in the same closed tube, the risk of the contamination of the environment by amplified nucleic acids is negligible. Automatic registration of an increase in the fluorescent signal during a positive reaction makes it possible to give a quantitative evaluation. The device's software issues the result immediately after the reaction.

RT-PCR has several advantages over culture-based technique. In contrast to the traditional culture-based technique, RT-PCR does not require special conditions for the transportation and storage of clinical samples (without compromising the quality of the test) and has high analytical sensitivity and specificity. It provides equal conditions for sensitivity and specificity for all the microorganisms in the sample, including non-culturable and difficult to culture. Moreover, the analysis time is much shorter than that of the culture-based method: less than one working day is required to carry out the test.

The sensitivity of RT-PCR in detecting easy-to-culture aerobic bacteria was 100% compared to cultures [16]. In the study of the pleural fluid of children with parapleural effusion/empyema, pathogens of the inflammatory process were identified by RT-PCR in 82% of cases, by culture method in 25% [18]. Horvath A. et al. (2013), comparing RT-PCR and culture methods for fungi, gram-positive and gram-negative bacteria, concluded that RT-PCR is a specific but faster method than culture-based methods [18]. Comparison of culture-based methods and RT-PCR showed 100% concordance in the diagnosis of such clinically significant bacteria as *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* [20]. It was concluded that RT-PCR is an accurate, fast and cost-effective laboratory method.

The combination of high sensitivity and specificity, low risk of contamination, the analysis time (1-1.5 hours), the possibility of quantitative analysis and identification of non-culturable microorganisms make RT-PCR technology highly demanded for the diagnosis of infectious diseases along with other laboratory research methods [21, 22].

Quantitative analysis of microorganisms by means of RT-PCR is currently being used to study normal, pathogenic and opportunistic microbiota in various human biotopes: periodontal [23], respiratory tract [24], samples from the gastrointestinal tract [25] and vaginal microbiota in women [26]. This approach was realized in kit "FEMOFLOR®" ("DNA-Technology", LLC) for evaluation of the microbiota of the urogenital tract

in women by RT-PCR [27-29]. Criteria for interpreting results of "Femoflor®" have been developed in several clinical trials with more than 2000 participants conducted in 2008–2011 [27-29]. The method has been certified and is successfully in routine use for the last 10 years.

Kit "Androflor®", "Androflor® Screen" ("DNA-Technology", LLC) was introduced recently for the evaluation of the male urogenital tract microbiota by RT-PCR.

Depending on the spectrum of detected microorganisms, the kit can be supplied in the following compositions:

- "Androflor®";
- "Androflor® Screen".

The "Androflor®" kit allows detecting the DNA of obligate pathogens, quantitative assessment of *Candida* fungi and opportunistic facultative and obligate anaerobes in order to give an etiological diagnosis of acute and chronic inflammatory diseases of the male urogenital system.

The Androflor® Screen kit is a short version of the Androflor® kit and is designed to detect DNA of obligate pathogens, perform a quantitative assessment of *Candida* fungi and shortened list of opportunistic microorganisms in order to give an etiological diagnosis of acute inflammatory diseases of the male urogenital system.

COLLECTION AND STORAGE OF SAMPLES

General requirements

To obtain correct results of RT-PCR, the preanalytical stage is of great importance: sample collection, storage and transportation of the sample to the laboratory.

The analysis of microorganisms' DNA by real-time PCR is a direct method of microbiological diagnostics, which is why the sample must be collected from the localizations closest to the suspected focus of the infectious process.

MATERIAL FOR THE TESTING

Swabs or scraping (from the glans penis, foreskin, preputial sac and urethra): recommended to diagnose acute and chronic infectious and inflammatory processes of the lower urinary tract.

Urine (first void urine specimen (FVU) or urine taken three hours after the last urination): recommended to diagnose acute inflammatory processes due to the pronounced painfulness of inserting the swab into the urethra.

Technique for collecting clinical samples for laboratory tests by RT-PCR method

1. Urethral specimen

Scraping is performed with a sterile disposable male urethral swab.

Before the collection of the specimen, the patient is recommended to refrain from urinating for at least three hours. In case of heavy urethral discharge urethral specimen is collected in 15–20 minutes after urination.

Immediately before collecting the sample, it is necessary to remove excess secretions from the opening of the urethra with a dry sterile cotton swab.

The urethral swab is inserted about 3–4 cm into the urethra and rotated in one direction for a minimum of 10 seconds.

In children, the urethral specimen is taken only from external orifice of urethra.

The urethral swab is inserted in a 1.5 ml Eppendorf tube with a transport medium for PCR testing. It is necessary to rinse the swab thoroughly in the transport medium, avoding splashing of the liquid, after which the swab is pressed against the wall of the tube, not covered with liquid, with a rotating motion.

Then, the swap has to be removed from the tube and the tube has to be tightly closed and labeled.

2. Scraping the epithelium of the foreskin, glans penis and preputial sac

Before the collection of the sample, the patient is recommended to refrain from urinating for 3-4 hours.

Scraping is performed with a sterile disposable male / female urethral swab from the surface of the foreskin, glans penis and the preputial sac.

The urethral swab is inserted in a 1.5 ml Eppendorf tube with a transport medium for PCR tests.

3. The procedure for collecting the first void urine specimen

Since the first morning urine sample is similar to scraping epithelial cells from the urethra, the first portion of morning urine is collected in the smallest possible volume (**several ml**) to increase the concentration of microorganisms in the sample. It is less preferable to use for testing the portion of urine collected after three or more hours after the last urination.

The urine is collected into a special dry sterile container with a volume of up to 60 ml equipped with a hermetically screwed cap.

After collecting the urine, container should be tightly closed and labeled.

CONDITIONS FOR STORAGE AND SAMPLE DELIVERY

In the accompanying document, it is necessary to specify the following: full name, age of the patient, type of material, preliminary diagnosis, indications for examination, date and time of collecting the sample, name of the institution (subdivision) sending the clinical material.

Clinical samples are delivered to the laboratory by persons who have received special instruction and in compliance with the rules of transportation.

If the storage time and transportation of clinical material from the moment of collecting until its delivery to the laboratory is no more than a day, then the tube with clinical material must be stored and delivered to the laboratory at the temperature 4–10°C.

When it is impossible to deliver the clinical sample to the laboratory within 24 hours, single freezing and storage of clinical samples is allowed at –20 °C for up to one month.

PERFORMING THE TEST

The isolation of nucleic acids from the sample and real-time PCR analysis are carried out at the laboratory according to the manufacturer's instructions.

INTERPRETATION OF TEST RESULTS

Results of PCR analysis are analyzed and interpreted automatically by the software (SW) designed for Real time PCR thermocyclers produced by DNA-Technology, LLC. Calculation of the total bacterial load (TBL) of the urethral microbiota, as well as absolute quantities of microorganisms in genome equivalents (GE), are carried out by the SW based on a mathematical formula that takes into account the number of the threshold cycle during RT-PCR. The SW calculates the relative amounts of a microorganism or a group of microorganisms as the difference between the absolute values of Lg10 of the microorganism/group of microorganisms and the TBL.

In order to generate the results, SW analyzes and evaluates the following parameters, depending on the configuration of the kit ("Androflor®" or "Androflor® Screen") (Table 1).

Table 1. Parameters of the Androflor® kit depending on the configuration.

INDICATORS	Androflor® Screen	Androflor®
Total bacterial load (TBL)	√	√
Human Genomic DNA (HGD)	√	√
Transient microbiota: *Lactobacillus spp.*	√	√
Normal microbiota		
Staphylococcus spp.	√	√
Streptococcus spp.	√	√
Corynebacterium spp.	√	√
Quantity: Normal microbiota	√	√
Opportunistic microorganisms (OM) associated with bacterial vaginosis (BV)		
Gardnerella vaginalis	√	√
Ureaplasma urealyticum	√	√
Ureaplasma parvum	√	√
Mycoplasma hominis	√	√
Atopobium cluster	–	√
Megasphaera spp./ Veillonella spp./ Dialister spp.	–	√
Sneathia spp./ Leptotrichia spp./ Fusobacterium spp.	–	√
Quantity: Opportunistic microorganisms associated with bacterial vaginosis (BV)	√	√
Opportunistic obligate anaerobes		
Bacteroides spp./ Porphyromonas spp./ Prevotella spp.	–	√
Anaerococcus spp.	–	√
Eubacterium spp.	–	√
Peptostreptococcus spp./ Parvimonas spp.	–	√
Quantity: Opportunistic anaerobes	–	√
OM: *Pseudomonas aeruginosa/ Ralstonia spp./ Burkholderia spp.*	–	√
OM: *Haemophilus spp.*	–	√
OM: *Enterobacteriaceae spp./ Enterococcus spp.*	√	√
Yeast-like fungi: *Candida spp.*	√	√

INDICATORS	Androflor® Screen	Androflor®
Pathogenic microorganisms		
Neisseria gonorrhoeae	√	√
Chlamydia trachomatis	√	√
Mycoplasma genitalium	√	√
Trichomonas vaginalis	√	√

Total bacterial load (TBL) shows the absolute quantity of bacteria in a specimen. The quantity of all detected bacteria / groups of bacteria are compared with TBL. The value clinically significant is $>10^4$ GE/ml.

Human genomic DNA (HGD) confirms the presence of human cells in the sample. HGD is contained in all human cells, except red blood cells. It is estimated in absolute values. The test is valid when HGD is $>10^3$ GE/ml.

If **both TBL$<10^4$ and HGD $<10^3$** in the specimen, the microbiota analysis could not be performed. The sampling must be repeated.

Transient microbiota: *Lactobacillus spp.* presence in male genital tract is considered as a marker of transient microbiota. It could origins from recent intercourse with female partner. But in some patients transient microbiota could also cause acute and chronic diseases of the lower male urogenital system. The clinically significant value is the proportion in TBL — it should not exceed 10% of the TBL. When the proportion of *Lactobacillus spp.* is >10% of TBL, the evaluation of microbiota is not carried out. The sampling must be repeated given that the patient has only protected sex or does not have unprotected sex within three days of collecting the sample.

Normal microbiota: *Staphylococcus spp., Streptococcus spp., Corynebacterium spp.* The total amount of microorganisms in the normal microbiota. This is a relative parameter the decrease of which is interpreted as dysbiosis.

Low TBL of the biotope (less than 10^4) is interpreted as NORMOCENOSIS given that the HGD is more than 10^3. If normal microbiota makes up most of the TBL, and the proportion of OM is below the clinically significant amounts, it is interpreted as NORMOCENOSIS.

If the proportion of normal microbiota is below the clinically significant amounts, it is interpreted as DYSBIOSIS, the severity of which is determined according to proportion of normal microbiota to the TBL. If the quantity of the TBL is 10^4–10^5, the severity of dysbiosis is not estimated due to possible mathematical errors. Then any decrease in the proportion of normal microbiota is interpreted as dysbiosis without specifying its severity.

Opportunistic microorganisms. The proportion of an OM and/or OM group in clinically significant amounts indicates their role in the dysbiosis. The etiology of dysbiosis is determined by comparing the proportions of specific OMs and/or OM groups. If one group of OMs prevails, it is identified as PREVALENT in the report. If there is no prevalent group of OMs, the dysbiosis is considered to be MIXED.

If the decrease in the proportion of normal microbiota is not associated with the increase in the tested OMs, it is interpreted as the DYSBIOSIS OF UNKNOWN ETIOLOGY.

Yeast-like fungi: *Candida spp*. The parameter is estimated in absolute values, the clinically significant amount is $>10^4$.

Pathogenic microorganisms: *Trichomonas vaginalis, Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium*. Qualitative parameter; the presence or absence of the microorganism is determined.

DESCRIPTION OF THE LAB REPORT

The structure of the "Androflor®" test lab report (Fig. 1) is presented, using a report of a specific patient. A lab report has the form of a table with three columns and a histogram.

The left column lists all the tested parameters.

The middle column (marked with purple in the Fig. 1) presents absolute values in the form of 10^n; boxes (colored and blank) correspond to the parameters that are determined in the absolute values.

The right column (marked with orange in the Fig. 1) presents relative values as follows: as the difference between Lg10 of the parameter and the TBL; as the proportion of the parameter in relation to the TBL (%). The difference is more mathematically correct, and the proportion is more commonly used. Boxes (colored and blank) correspond to the parameters that are evaluated in the relative values.

The histogram (marked with blue in the Fig. 1) presents all the quantitative parameters. The bars are color coded in accordance with the influence a particular parameter has on the results. % of the TBL are given at the top of the histogram, and Lg10 values are given in the bottom.

The colors of the boxes in the table mean the following:

Controls

(HGD, TBL, *Lactobacillus spp.*):

- ☐ valid,
- ■ invalid.

Normal microbiota:

- ■ norm,
- ■ moderate deviation from the norm,
- ■ apparent deviation from the norm.

OM and yeast-like fungi:

- ☐ norm,
- ■ moderate deviation from the norm,
- ■ apparent deviation from the norm.

Pathogenic microorganisms:

- ☐ not detected,
- ■ detected.

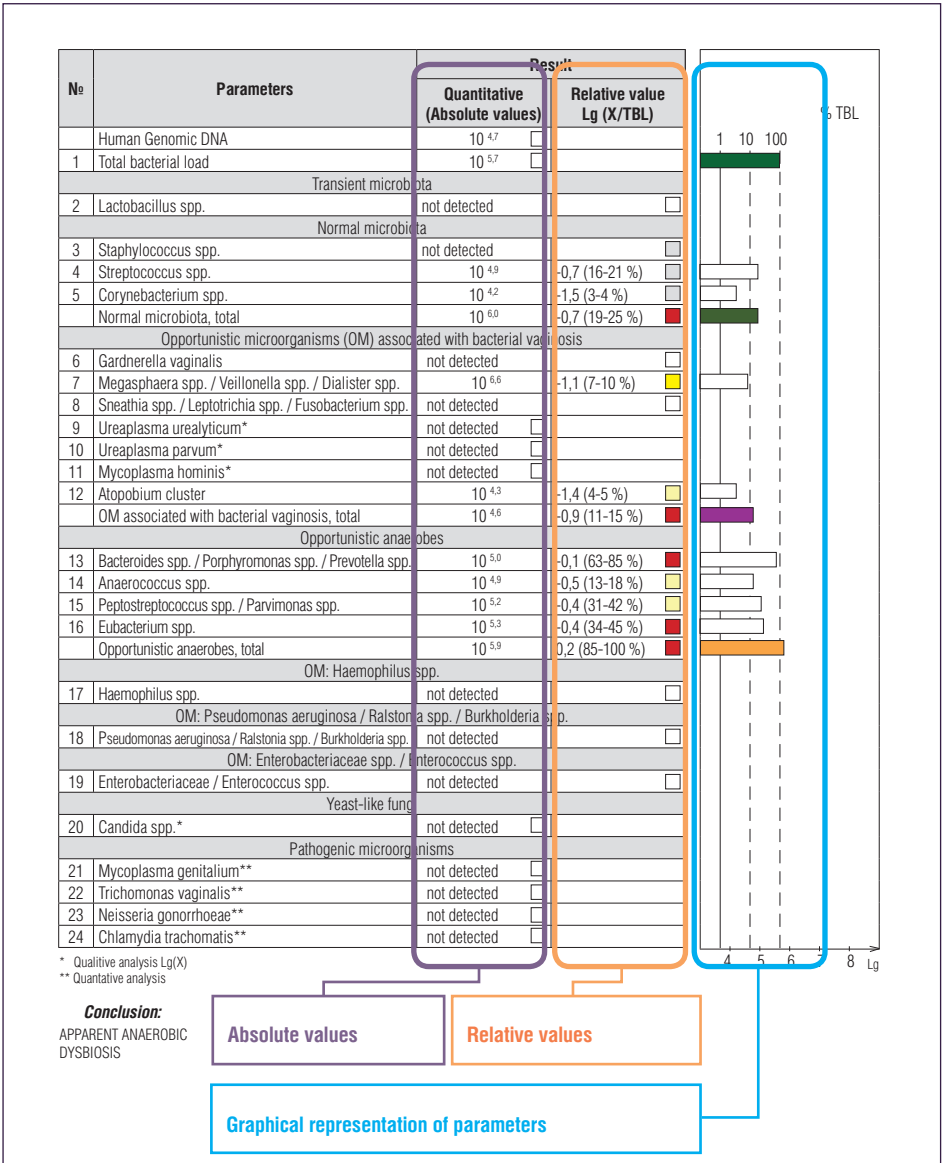


Fig. 1. The structure of the lab report of the Androflor® kit, generated automatically by the software supplied with the Real time PCR thermocyclers manufactured by DNA-Technology.

To give a more detailed description of the structure, the lab report was divided into four parts (Fig. 2–5).

Figure 2 shows the 1st part of the lab report with the following parameters:

"Human genomic DNA" is a control absolute parameter where the value $10^{4,7}$ meets the criteria of the norm. The box is blank which means that HGD is present in the sample.

"Total bacterial load" is a control absolute parameter where the value of $10^{5,7}$ meets the criteria of the norm. The box is blank, which indicates sufficient quantity of TBL (more than 10^5) to assess the severity of dysbiosis.

"Transient microbiota": *Lactobacillus spp.* is a control relative indicator, the result is "not detected", which meets the criteria of the norm, the box is blank.

"Total: Normal microbiota" is a relative parameter, –0.7 Lg10 (19–25%) of the TBL, which is a pronounced deviation from the norm. The box is red, and the color of the bar suggests that this parameter has an impact on the results.

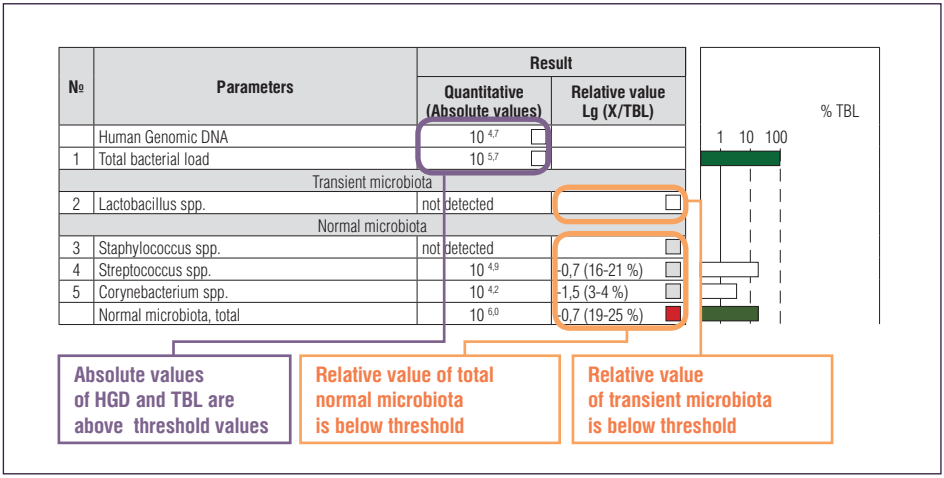


Fig. 2. Description of the lab report, part 1.

Figure 3 shows the 2nd part of the lab report with the following parameters:

"Opportunistic microorganisms associated with bacterial vaginosis (BV)" includes absolute and relative values. Genital mycoplasmas are evaluated in absolute values, all other OM — in relative values.

Genital mycoplasmas were not detected, the box is blank. Among the remaining OM, two parameters moderately deviate from the norm — the boxes are yellow. The proportion

of the sum of all “OMs associated with BV” including genital mycoplasmas, significantly deviates from the norm: –0.9 Lg10 (11–15%) of the TBL, the box is red. The histogram bar is colored.

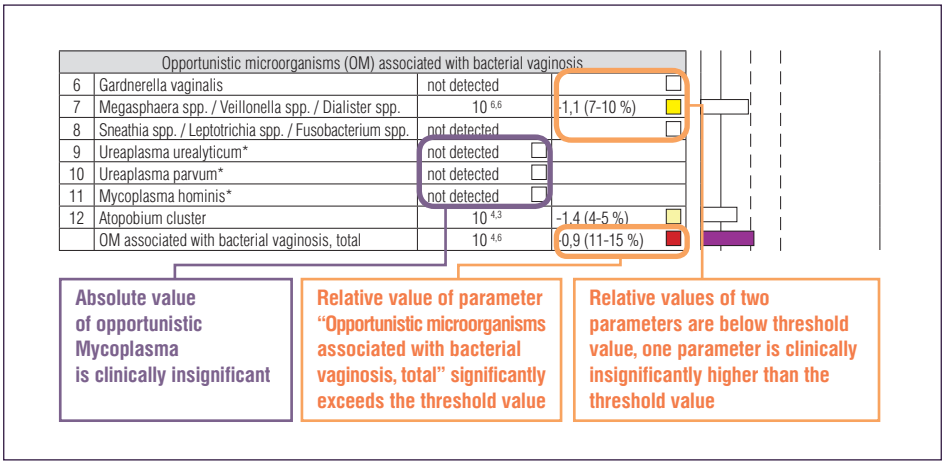


Fig. 3. Description of the lab report, part 2.

Figure 4 shows the 3d part of the lab report with the following parameters:

“Opportunistic anaerobes” is estimated in relative values. Two OM groups significantly deviate from the norm criteria (the boxes are colored red), two OM groups moderately deviate from the norm (the boxes are colored yellow). The parameter “Total: Opportunistic anaerobes” also significantly deviates from the norm (the box is red), the histogram bar corresponding to this parameter is colored because it influences the results of the test.

“OM *Haemophilus spp.*”, “OM *Pseudomonas aeruginosa / Ralstonia spp. / Burkholderia spp.*”, “OM *Enterobacteriaceae / Enterococcus spp.*” are relative parameters, microorganisms are not detected, meeting the criteria of the norm, the boxes are blank.

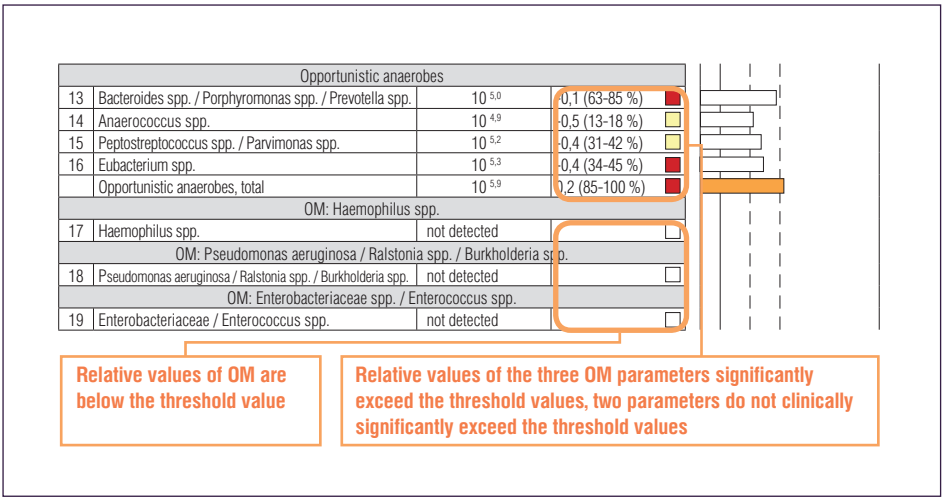


Fig. 4. Description of the lab report, part 3.

Figure 5 shows the 4th part of the lab report with the following parameters:

"Yeast-like fungi", *Candida spp.* — absolute parameter, the result "not detected" meets the criteria of the norm, the box is blank.

"Pathogenic microorganisms", *Trichomonas vaginalis, Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium* — a qualitative parameter, the presence or absence of the pathogen is tested, none of the pathogens are detected, meeting the criteria for the norm, the boxes are blank.

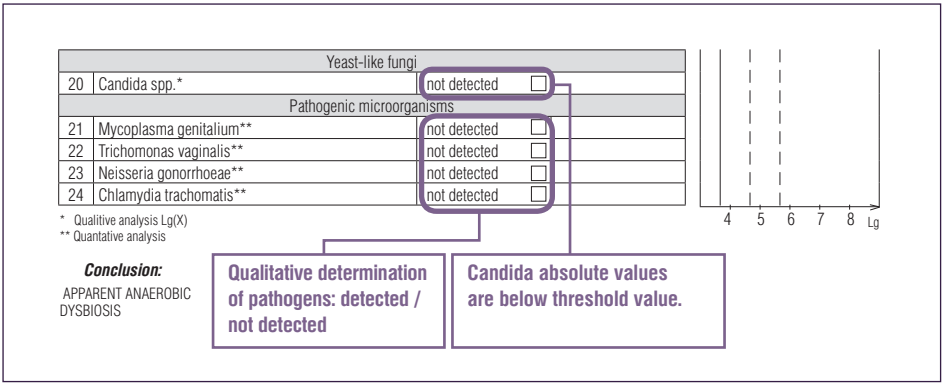


Fig. 5. Description of the lab report, part 4.

In accordance with the developed algorithm (application), the thermocycler's software automatically generated the result "APPARENT ANAEROBIC DYSBIOSIS" (Fig. 6) based on the pronounced deviation from the criteria of the norm of the "Total: Normal microbiota" parameter. The conclusion "anaerobic" is formed on the basis of the parameter "Total: Opportunistic anaerobes" which significantly exceeds the parameter "Total: OMs associated with bacterial vaginosis".

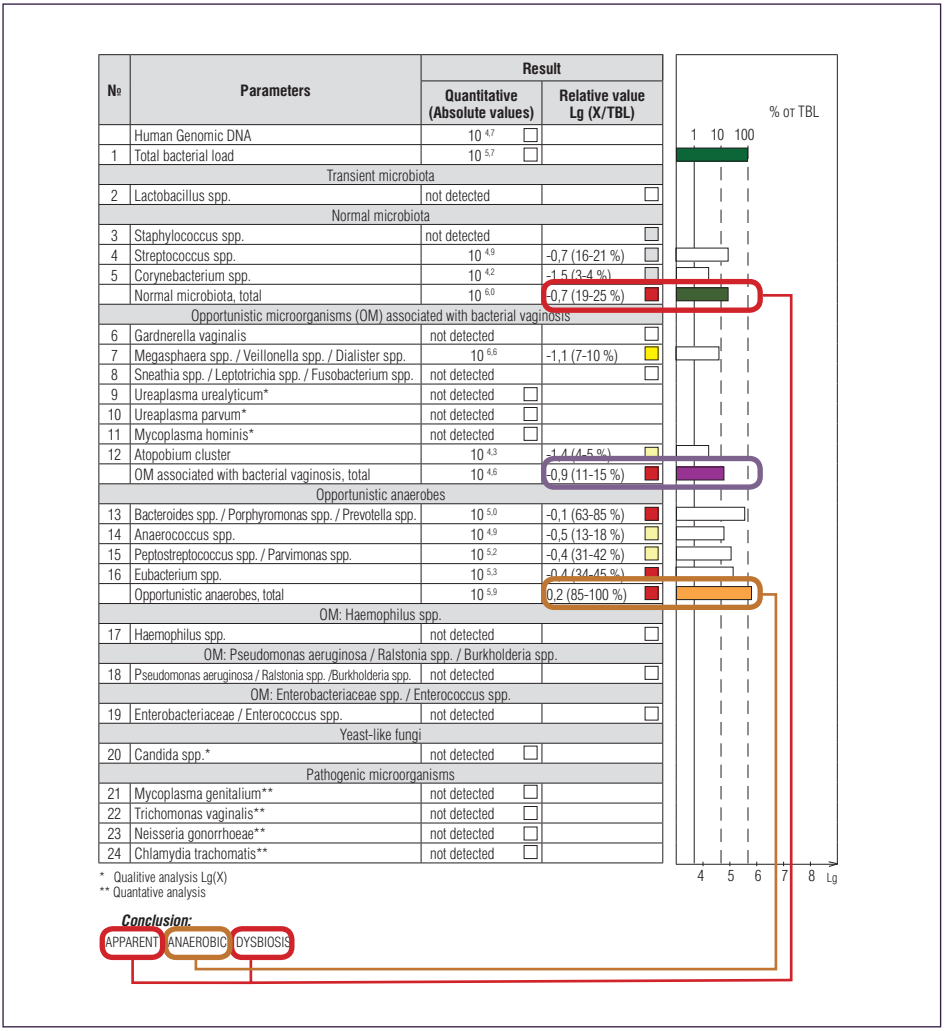
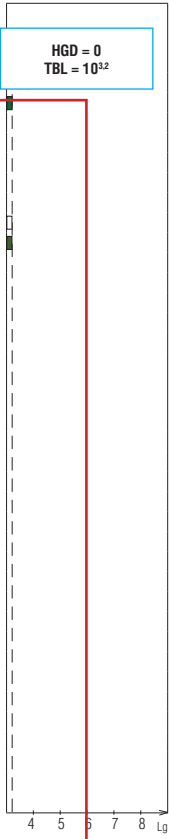


Fig. 6. An example of a lab report.

EXAMPLES of lab reports with generated after analysis of male urogenital microbiota using the RT-PCR Kit “Androflor®”

№	Parameters	Result	
		Quantitative (Absolute values)	Relative value Lg (X/TBL)
	Human Genomic DNA		
1	Total bacterial load	$10^{3,2}$	
	Transient microbiota		
2	Lactobacillus spp.	not detected	☐
	Normal microbiota		
3	Staphylococcus spp.	not detected	☐
4	Streptococcus spp.	not detected	☐
5	Corynebacterium spp.	$10^{2,1}$	0,0 (64-100 %) ☐
	Normal microbiota, total	$10^{2,1}$	0,0 (64-100 %) ■
	Opportunistic microorganisms (OM) associated with bacterial vaginosis		
6	Gardnerella vaginalis	not detected	☐
7	Megasphaera spp. / Veillonella spp. / Dialister spp.	not detected	☐
8	Sneathia spp. / Leptotrichia spp. / Fusobacterium spp.	not detected	☐
9	Ureaplasma urealyticum*	not detected ☐	
10	Ureaplasma parvum*	not detected ☐	
11	Mycoplasma hominis*	not detected ☐	
12	Atopobium cluster	not detected	☐
	OM associated with bacterial vaginosis, total	not detected	☐
	Opportunistic anaerobes		
13	Bacteroides spp. / Porphyromonas spp. / Prevotella spp.	not detected	☐
14	Anaerococcus spp.	not detected	☐
15	Peptostreptococcus spp. / Parvimonas spp.	not detected	☐
16	Eubacterium spp.	not detected	☐
	Opportunistic anaerobes, total	not detected	☐
	OM: Haemophilus spp.		
17	Haemophilus spp.	not detected	☐
	OM: Pseudomonas aeruginosa / Ralstonia spp. / Burkholderia spp.		
18	Pseudomonas aeruginosa / Ralstonia spp. /Burkholderia spp.	not detected	☐
	OM: Enterobacteriaceae spp. / Enterococcus spp.		
19	Enterobacteriaceae / Enterococcus spp.	not detected	☐
	Yeast-like fungi		
20	Candida spp.*	not detected ☐	
	Pathogenic microorganisms		
21	Mycoplasma genitalium**	not detected ☐	
22	Trichomonas vaginalis**	not detected ☐	
23	Neisseria gonorrhoeae**	not detected ☐	
24	Chlamydia trachomatis**	not detected ☐	



* Qualitive analysis Lg(X)
** Quantitative analysis

Conclusion: Insufficient amount of biomaterial, it is recommended to repeat the sampling

Fig. 7. Case 1. “Insufficient amount of biomaterial, it is recommended to repeat the sampling”.

The conclusion is based on the fact that the two control parameters, HGD and TBL do not meet the required criteria, i.e. the sample has no epithelial cells (containing human DNA).

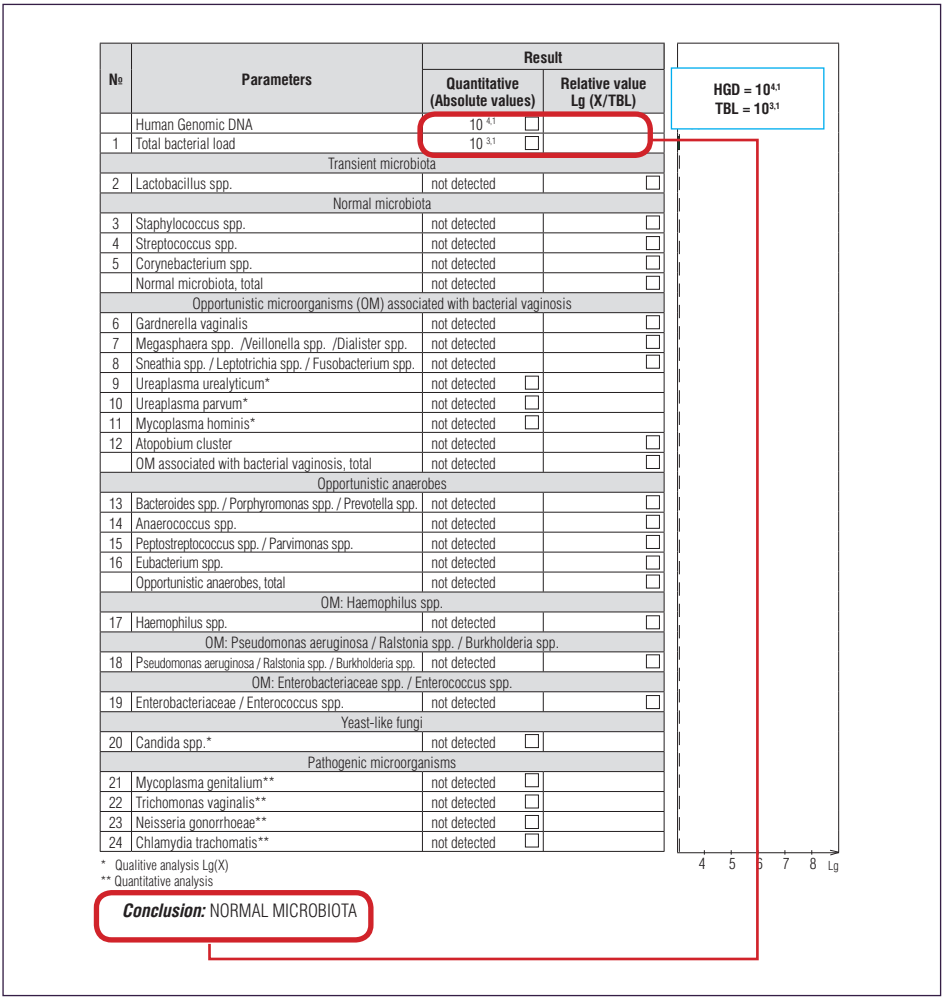


Fig. 8. Case 2. "Normal microbiota".

HGD parameter in this example meets the required criteria (the sample contains epithelial cells with human DNA); TBL parameter in this test is too low to analyze the microbiota structure. Low TBL of the urogenital specimen is considered to be the normal variant given the lack of sexual contacts.

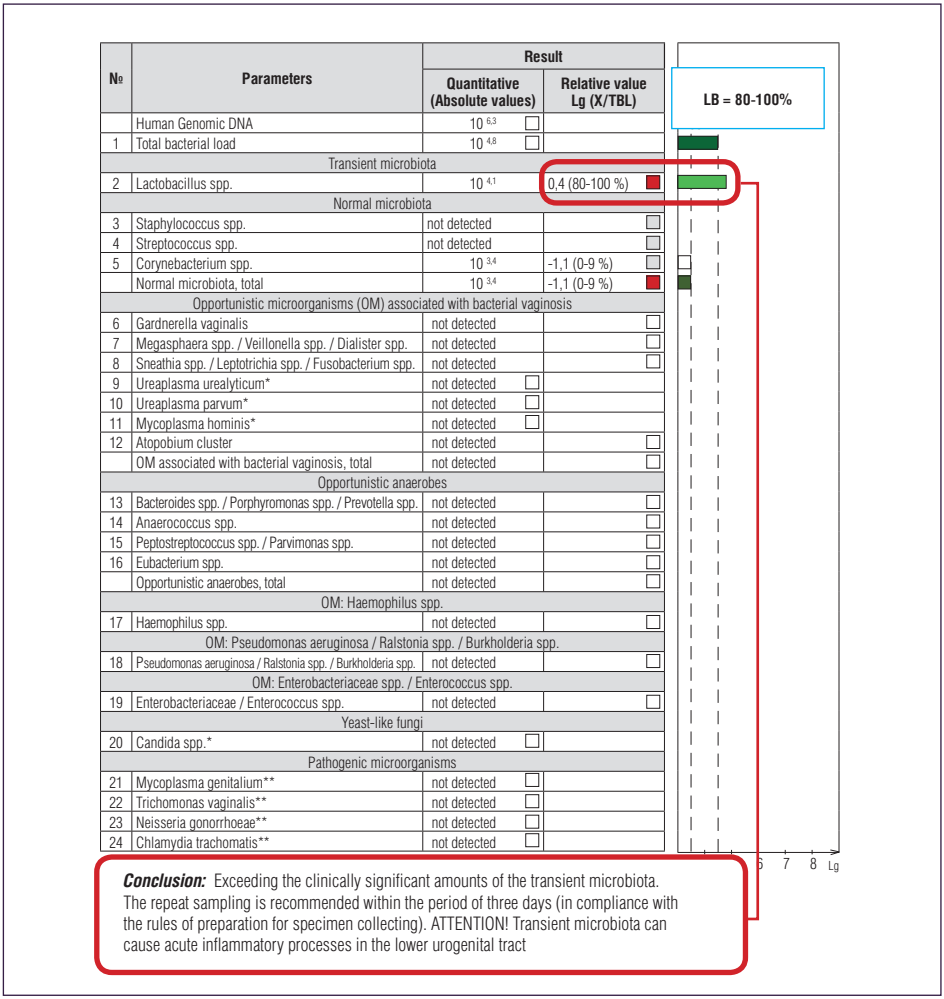


Fig. 9. Case 3. "Exceeding the clinically significant amounts of the transient microbiota. The repeat sampling is recommended within the period of three days (in compliance with the rules of preparation for specimen collecting). ATTENTION! Transient microbiota can cause acute inflammatory processes in the lower urogenital tract".

The conclusion is based on the fact that the parameter "Transient microbiota: *Lactobacillus spp.*" (LB) does not meet the required criteria, i.e. exceeds the accepted value. The analyses of male microbiota structure is impossible.

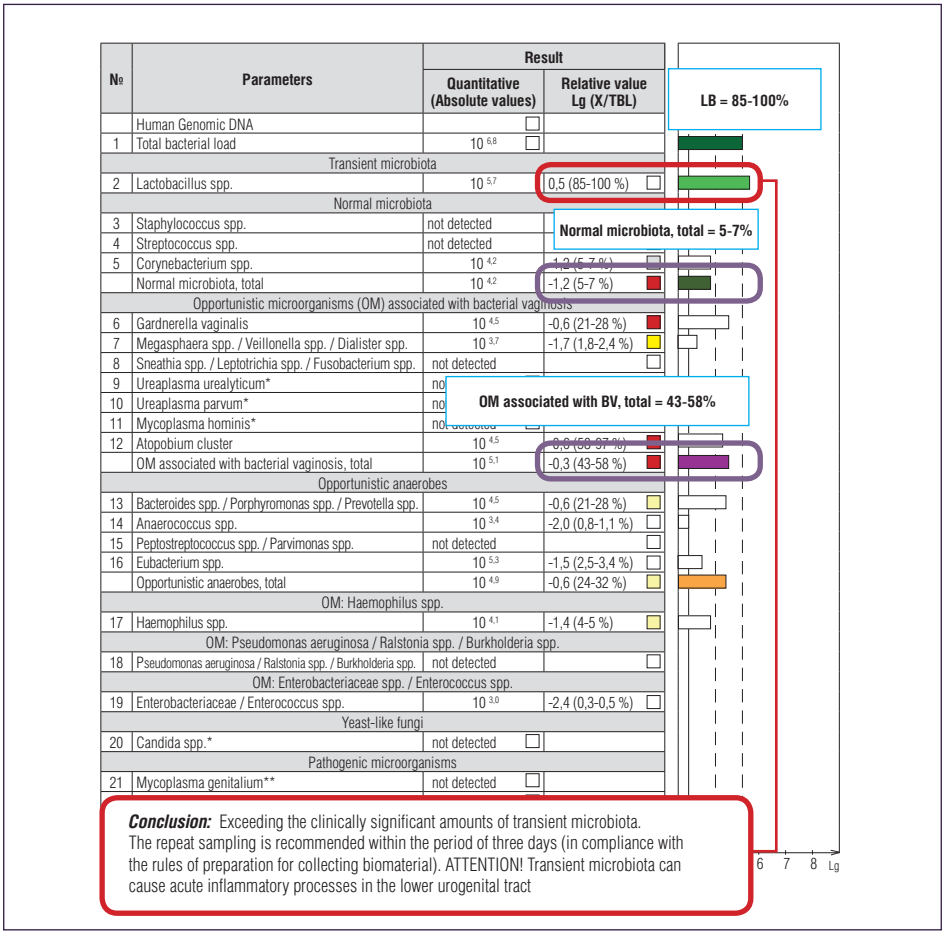


Fig. 10. Case 4. "Exceeding the clinically significant amounts of transient microbiota. The repeat sampling is recommended within the period of three days (in compliance with the rules of preparation for collecting biomaterial). ATTENTION! Transient microbiota can cause acute inflammatory processes in the lower urogenital tract".

The parameter "Transient microbiota: *Lactobacillus spp.*" (LB) does not meet the required criteria. The relative parameter "Total: OMs associated with bacterial vaginosis" is 43–58% which significantly differs from the norm; however, high amounts of transient microbiota make it impossible to form a conclusion about the structure of the patient's microbiota. In this case, transient microbiota can be the cause of an acute disease of the lower urogenital system.

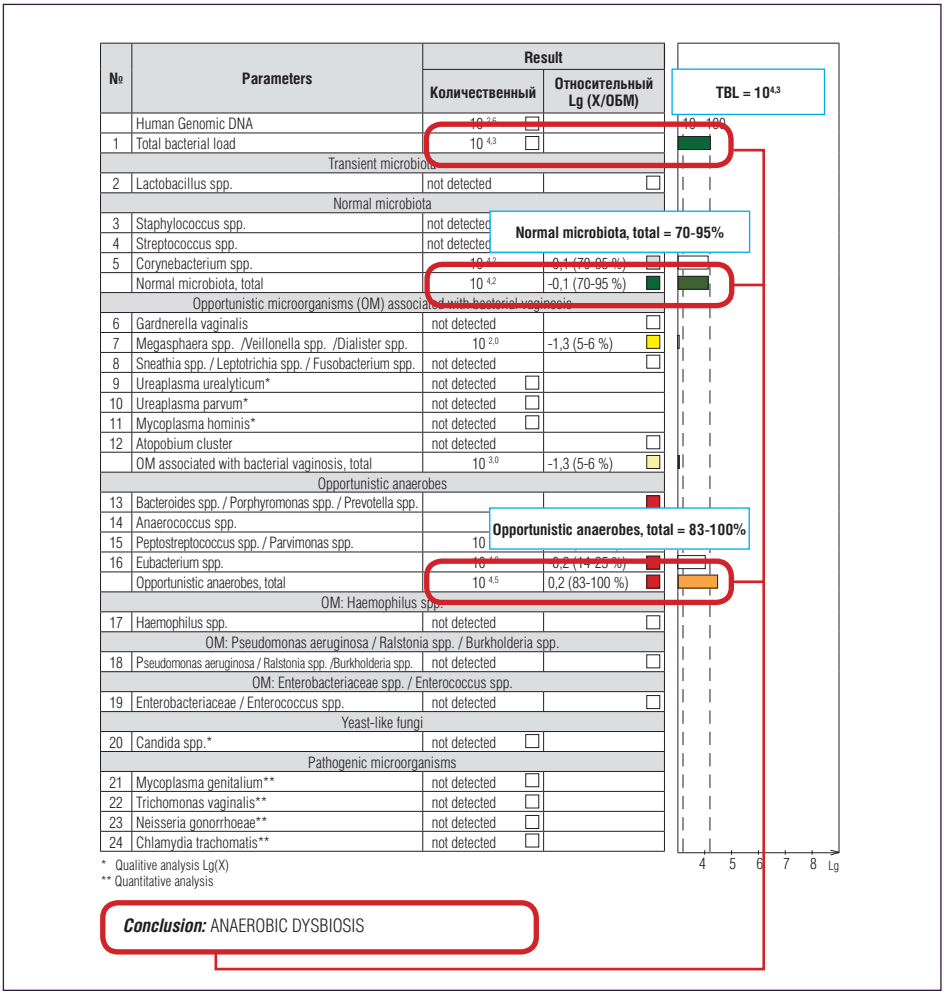


Fig. 11. Case 5. "Anaerobic dysbiosis".

TBL = 4.3, i.e. is in the range of 4.0–5.0 lg10; the assessment of the dysbiosis severity is not performed due to possible mathematical errors. The value of the only parameter "Total: Opportunistic anaerobes" significantly exceeds the criteria of the norm, therefore the results are interpreted as **anaerobic dysbiosis**.

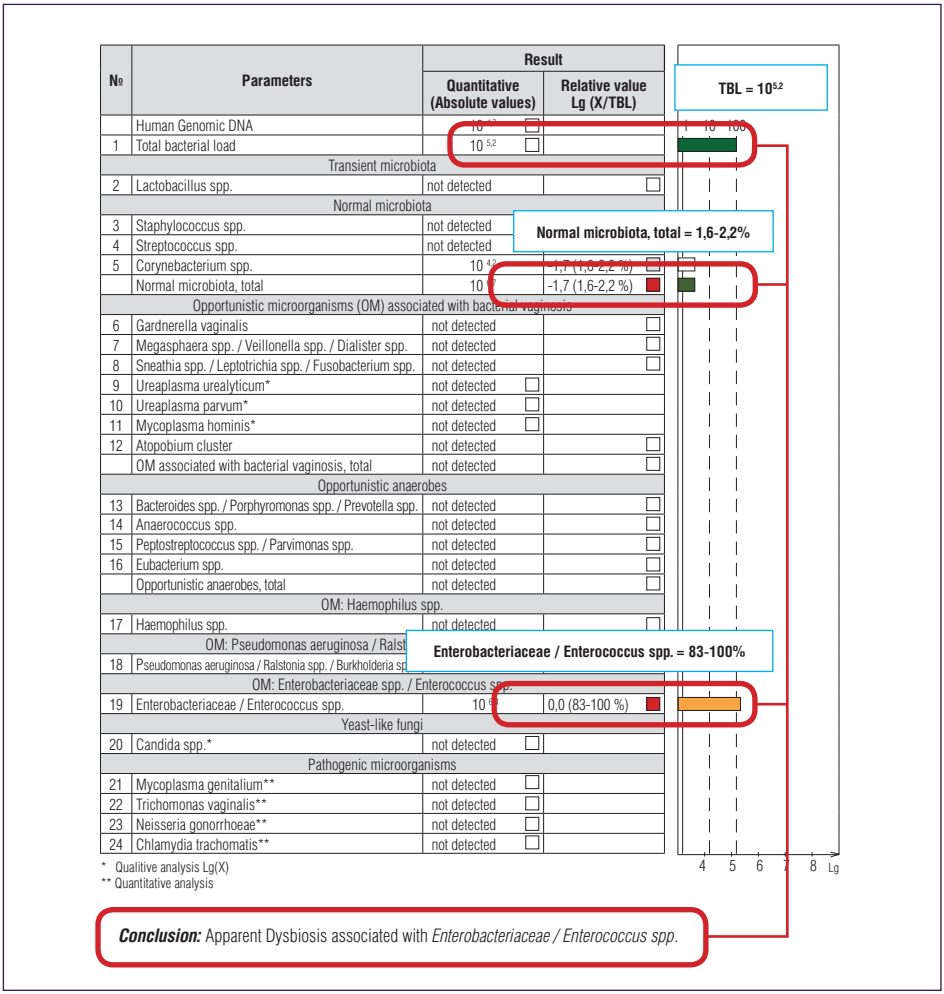


Fig. 12. Case 6. "Apparent Dysbiosis associated with *Enterobacteriaceae / Enterococcus spp.*".

TBL >10^5, i.e. meets the criteria for assessing the degree of dysbiosis with a decreased normal microbiota. The value of the "Total: Normal microbiota" parameter is significantly low, therefore the results are interpreted as **apparent dysbiosis**. Since the value of the single parameter, "OM *Enterobacteriaceae / Enterococcus spp.*", significantly deviates from the norm, the dysbiosis is considered to be associated with *Enterobacteriaceae / Enterococcus spp.*".

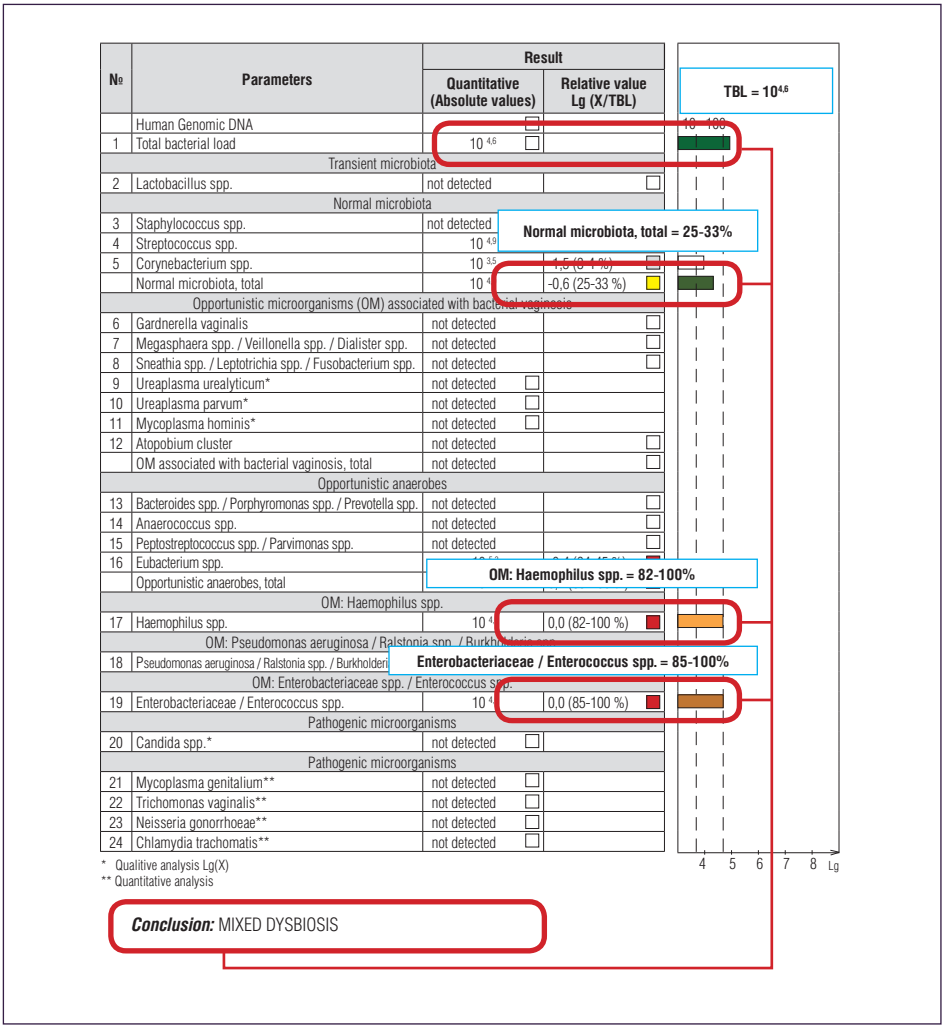


Fig. 13. Case 7. "Mixed dysbiosis".

Since TBL is in the range of 4.0–5.0 lg10, the severity of dysbiosis is not determined due to a possible mathematical error. The value of the "Total: Normal microbiota" parameter is moderately below the norm. The values of two parameters (*Haemophilus spp., Enterobacteriaceae/ Enterococcus spp.*) significantly deviate from the norm and neither could be considered prevalent, so the conclusion is **mixed dysbiosis**.

V. CLINICAL TRIALS

173 men with urogenital complaints underwent clinical and laboratory examination in the period of 2009–2015; 61 asymptomatic men were included in the control group, age range 18–45.

General exclusion criteria: somatic diseases in the stage of decompensation, oncological diseases, endocrinopathy, receiving systemic antimicrobial treatment within two months before the examination, the use of topical treatment within three weeks examination, syphilis, HIV, hepatitis B, C.

All study participants were informed about the purpose of the study and gave written consent to participate in the clinical and laboratory examination.

The urethral specimens were obtained with disposable sterile urethral swabs and placed in an Eppendorf tube with a transport medium.

The material was analyzed using real-time PCR with Androflor® kit ("DNA-Technology", LLC, Moscow).

Statistical data processing and the construction of the ROC curve were performed using the SPSS statistical software package (SPSS Inc., Chicago, USA). The median of absolute and relative amounts of microorganisms/groups of microorganisms were calculated in order to describe microbiota in the study groups. Upper and lower quartiles were used as a measure of dispersion. Comparison of groups was performed using nonparametric statistics (Manni — Whitney test).

CLINICAL RESULTS

General characteristics of the control group

The control group included 61 men who sought medical care due to infertility in a couple (female infertility was revealed). Men were included in the control group if the results of clinical and laboratory examination did not reveal symptoms of inflammation and STI pathogens.

Inclusion criteria: aged from 18 to 45 years, no urogenital complaints, no history of urogenital tract diseases, no unprotected sex within at least three days before collecting the specimen, no sexually transmitted pathogens detected by PCR.

General characteristics of the experimental group of patients

Inclusion criteria: aged from 18 to 45 years, urogenital complaints, clinical signs of lower urogenital tract diseases of different severity, the presence (n = 42) or absence (n = 131) of sexually transmitted pathogens (*Trichomonas vaginalis, Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium*) detected by PCR.

A total of 173 (n = 100%) men aged 18 to 45 years were examined.

Clinical characteristics of the experimental group of patients depending on the presence (STI+) or the absence of STIs (STI-)

Distribution of complaints and clinical symptoms were compared in two groups of patients formed on the basis of the presence or absence of sexually transmitted pathogens.

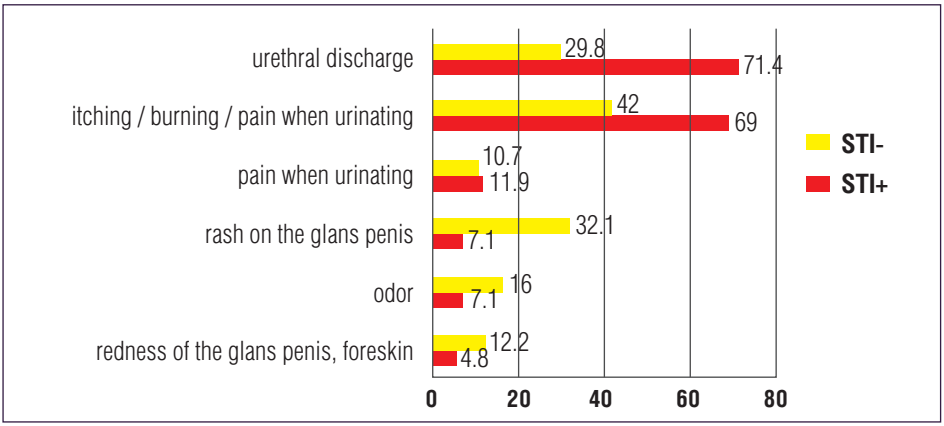


Fig. 14. Comparative analysis of the incidence (%) of urogenital complaints in the groups of men with the presence of STIs and the absence of STIs (GP — glans penis; FS — foreskin).

Complaints of dysuria were more common among STI+ men. At the same time, a rash on the glans penis was found more often in the group of STI- men (Fig. 14)

The results of clinical examination also differed between the groups (Fig. 15). Rashes and plaque were found more often in STI- patients (29% against 4.8%, $p < 0.01$ and 14.5% against 7.6%, $p < 0.01$ respectively).

The nature of the urethral discharge in two groups also differed: opaque and non-mucous secretions were more specific to the STI+ group (31% versus 7.6% $p < 0.01$), as well as higher amount of discharge — profuse (16.7% vs. 3,8%, $p < 0.01$)

or moderate (26.2% vs. 8.4%, $p < 0.01$) compared to patients of the STI- group. Discharge was clear (36.6% versus 14.3%, $p < 0.01$) and in small quantity (25.2% versus 7.1%, $p < 0.01$) in the STI- group.

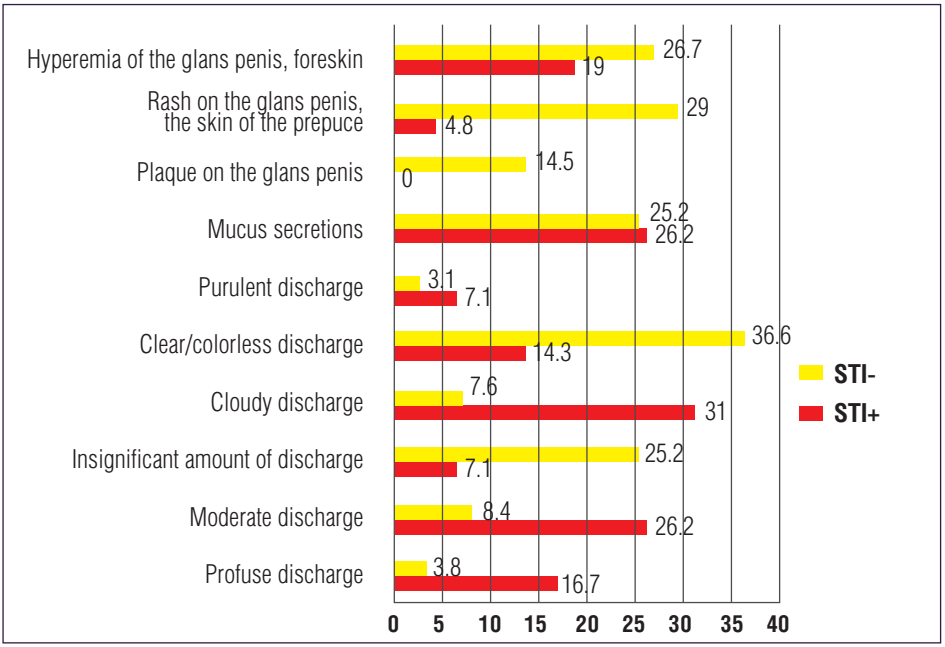


Fig. 15. Comparative analysis of the incidence (%) of objective clinical symptoms of inflammation in the STI+ and STI- groups.

Clinical diagnosis also differed in these two groups of patients (Fig. 16): the most frequent diagnosis in the STI+ group was acute urethritis (85.7% vs. 35.1%, $p < 0.01$), while in the STI- group — acute balanoposthitis (45.8% versus 9.5, $p < 0.01$) and chronic urethritis (11.5% versus 0, $p < 0.01$).

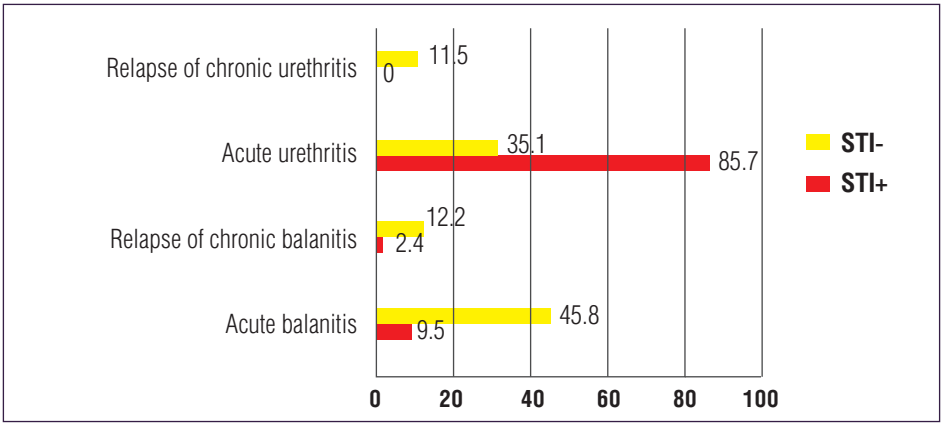


Fig. 16. Distribution (%) of clinical diagnoses in groups of patients with and without STIs.

RESULTS OF LABORATORY TESTS

Distribution of patients depending on the detected pathogens of STIs

In most cases, Chlamydia trachomatis was detected (n = 26; 61.9%) in STI+ patients (n = 42). *Mycoplasma genitalium* was detected in 10 (23.8%) patients, *Neisseria gonorrhoeae* — in 5 (11.9%) and *Trichomonas vaginalis* — in 1 (2.3%) patient.

Microbiota in patients with STIs compared to the control group

TBL in 6 men from the control group was below 10^4 GE/ml. Comparative data on TBL of the urethra in patients with STIs and in the control group are presented in Table 2. TBL in patients with STI+ was significantly higher than in the control group. The highest quantity of TBL was detected in patients with *Neisseria gonorrhoeae*. In patients infected by *Chlamydia trachomatis* or *Mycoplasma genitalium*, the quantity of TBL was lower. In the only patient with trichomoniasis, TBL was $10^{6.1}$ GE/ml.

Table 2. TBL of the urethra in the control group and in STI+ patients (lg10).

Patient groups	Median	Quartile		P (compared to the control group)	P (compared to *N. gonorrhoeae*)
		25	75		
Control group	4.7	4.4	5.3		
Neisseria gonorrhoeae	6.4	6.0	7.2	.000	
Chlamydia trachomatis	5.7	5.2	6.1	.000	.019
Mycoplasma genitalium	5.5	5.3	6.3	.000	.028
All STIs	5.8	5.4	6.3	.000	

Compared to the control group the most pronounced changes in the microbiota structure of STI+ patients are a significant decrease in the proportion of normal microbiota (*Staphylococcus spp., Streptococcus spp., Corynebacterium spp.*) and of the "Total: Normal microbiota" parameter (p = 0.0000) in addition to an increase in TBL.

According to ROC analysis the parameter "Total: Normal microbiota" distinguishes the control group from the STI+ group of patients (area under the ROC curve = 0.93; 0.87–1.0, p = 0.000) (Fig. 17).

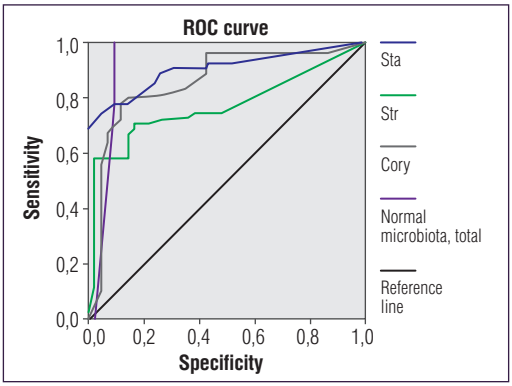
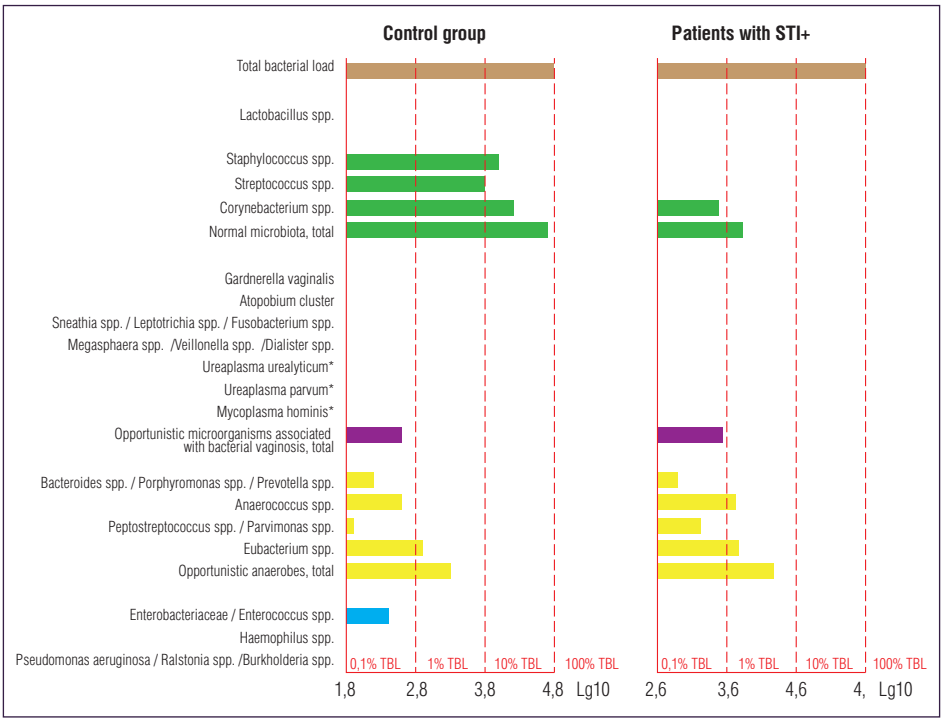


Fig. 17. ROC curves for diagnostic tests that distinguish the control group from the STI+ group.

Other differences in the structure of the microbiota between the two groups were not so strong (Fig. 18). The proportion of obligate-anaerobic OM (non-culturable *Anaerococcus spp., Peptostreptococcus spp./ Parvimonas spp. and Eubacterium spp.*) were slightly but clinically significantly increased in STI+ patients compared to the control group.

Higher quantities of anaerobes in STI+ patients could suggest that this type of "background" microbiota increases the risk of getting STI.



A. Control group ***B. Patients with STI***

Fig. 18. Microbiota in male patients with subjective and objective symptoms of the lower urogenital system inflammation and in STI+ patients.

Microbiota in patients without STIs compared to the control group

Microbiota of patients with a primary episode of acute balanitis was not significantly different from the microbiota of patients with a primary episode of acute urethritis. Based on this, patients with primary episodes of the lower urogenital tract infection (balanitis, urethritis, a combination of balanitis and urethritis) were combined into one group (patients with acute lower urinary tract symptoms (LUTS) (n = 101)).

Microbiota of patients with recurrent episodes of chronic balanitis was not significantly different from the microbiota of patients with recurrent episodes of chronic urethritis. Thus, patients with recurrent episodes of the lower urinary tract infection (balanitis, urethritis, a combination of balanitis and urethritis) were combined into one group of patients with chronic LUTS (n = 30).

Using the same logic, STI- patients with acute and chronic LUTS were combined into one group of patients (n = 131) with the lower urinary tract diseases.

TBL in the lower urinary tract in STI- patients with acute and chronic diseases was approximately 10 times higher than in the control group (Me = 5.6, 4.9-6.3lg10 vs. Me = 4.7 4.4-5.3 lg10, p = 0.000), and was not significantly different from the quantity of TBL in STI+ patients (Me = 5.8, 5.4-6.3 lg10, p > 0.05).

In STI- patients with the urogenital tract diseases, as well as in STI+ patients with the urogenital tract diseases, a significant decrease in the proportion of normal microbiota was found: *Staphylococcus spp., Streptococcus spp., Corynebacterium spp.* and in the "Total: Normal microbiota" parameter (p = 0.000).

Similarly to STI+ group, "Total: Normal microbiota" parameter distinguishes the control group from the group of STI- patients with diseases of the urogenital tract with the highest accuracy, according to ROC analysis (area under the ROC curve = 0.93; 0.89–0.97, p = 0.000) (Fig. 19).

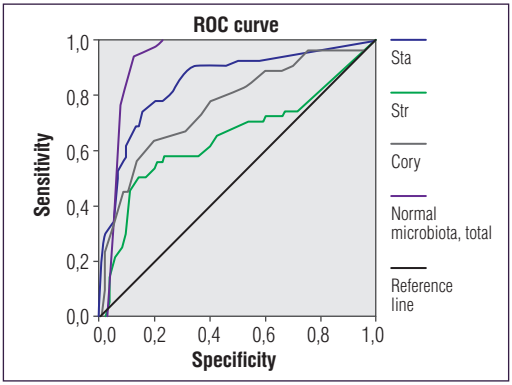


Fig. 19. ROC curves for diagnostic tests that best distinguish the control group from the group of STI- patients.

In addition to a decrease in the proportion of normal microbiota (Fig. 20), a small but significant increase in the proportion of transient microbiota (*Lactobacillus spp*.) was detected (p = 0,016) in STI- patients with LUTS, unlike in STI+ patients. This indicates the possible involvement of the transient microbiota in the development of diseases of the lower urogenital tract in some patients.

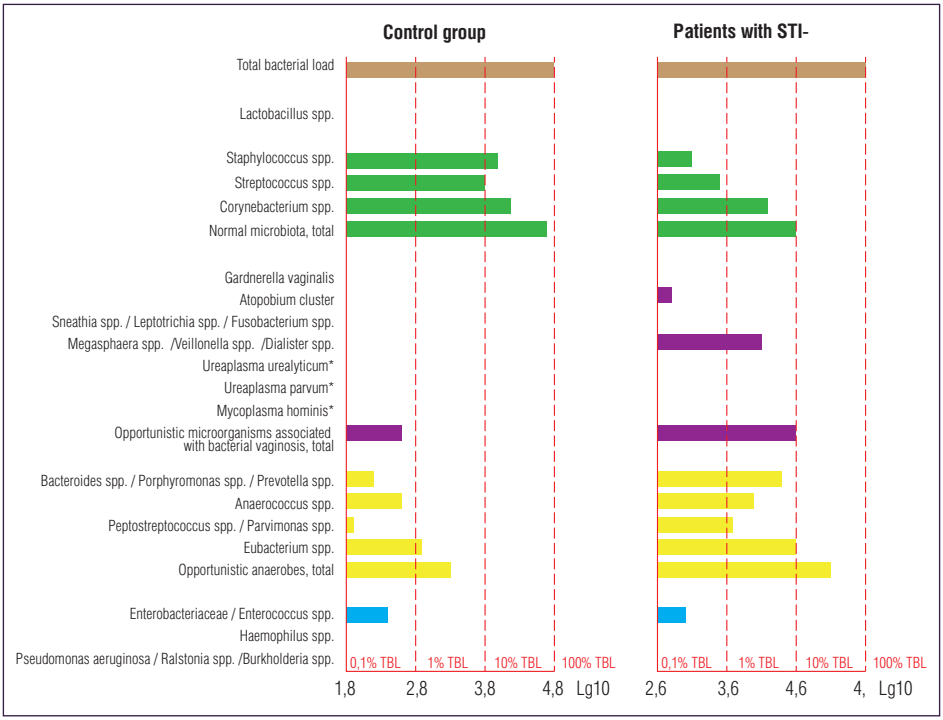
Also, an increase in the proportion of some groups of opportunistic microorganisms was detected in STI- patients compared with the control group. This refers to two groups of OM: bacteria associated with BV and obligate anaerobes.

Among the OMs associated with BV, the proportion of *Gardnerella vaginalis* (p = 0.000), *Megasphaera spp./ Veillonella spp./ Dialister spp*. (p = 0.000), *Sneathia spp./ Leptotrichia spp./ Fusobacterium spp.* (p = 0.008) and absolute quantities of *U. urealyticum* (p = 0.007), *U. parvum* (p = 0.001), and *M. Hominis* (p = 0.027) were significantly increased in STI- patients compared to the control group. At the same time, the proportion of patients with opportunistic genital mycoplasmas was small: U. urealyticum in quantities greater than 10^4 GE/ml was detected in 13 patients (9.9%), *U. parvum* — in 10 patients (7.6%), and *M. hominis* — in 7 patients (5.3%). The proportion of the parameter "Total: OMs associated with bacterial vaginosis", which includes the Atopobium cluster in addition to the listed parameters, was also increased in STI-patients compared to the control group (p = 0.000).

Among the anaerobes, the proportion of *Bacteroides spp. / Porphyromonas spp. / Prevotella spp.* (0.000), *Anaerococcus spp.* (0.000), *Eubacterium spp.* (0.000), P*eptostreptococcus spp. / Parvimonas spp.* (0.000) and the value of the parameter "Total: OM anaerobes", which includes all the listed microorganisms, were significantly increased in STI- patients compared to the control group.

The proportion of *Haemophilus spp*. was also significantly increased in STI- patients compared to the control group (p=0.003).

Only the following relative parameters of the Androflor® kit were similar in STI-patients and in patients from the control group: *Atopobium cluster, Pseudomonas aeruginosa/ Ralstonia spp./ Burkholderia spp.* and *Enterobacteriaceae spp./ Enterococcus spp.* The absolute quantities of Candida spp were similar in patients from these two groups.



A. Control group ***B. Patients with STI***

Fig. 20. The microbiota composition in male patients with subjective and objective symptoms of the lower urinary tract inflammation without STIs (B) and the control group (A).

To assess whether the diagnostic parameters included in the Androflor® kit can correctly classify patients with clinical symptoms of lower urogenital tract diseases in STI- patients and a control group, as well as to select parameters that differentiate both groups in the best possible way, ROC analysis was performed (Fig. 21). As a result, the highest diagnostic accuracy, which is regarded as good, was demonstrated by two parameters: “Total: OMs, associated with bacterial vaginosis” (AUC = 0.84, 0.72–0.90, p = 0.000) and “Total: OM anaerobes ”(AUC = 0.87, 0.83–0.93, p = 0.000).

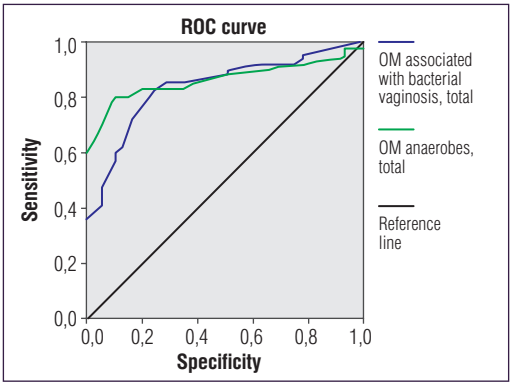


Fig. 21. ROC curves corresponding to diagnostic tests with the highest accuracy distinguishing a group of patients from a control group ("Total: OMs, associated with bacterial vaginosis" and "Total: OM anaerobes").

To determine which combination of diagnostic parameters best differentiates STI- patients from the control group a discriminant analysis was performed. The greatest accuracy of distinguishing between the STI- patients and patients from the control group was observed when using three classifying parameters: "Total: Normal microbiota", "Total: OM associated with bacterial vaginosis" and "Total: OM anaerobes", and amounted to 91.4%.

SUMMARY

Clinical symptoms of the lower urogenital tract disorders are more severe, and urethral lesions are more frequent in the STI+ patients compared to the STI- patients. However, these differences are not absolute.

Etiology of the urogenital system disorders in STI- patients is complex. Clinical symptoms are associated with an increased TBL in urethra mainly through the increased quantities of different OMs.

Both in STI+ and STI- patients TBL in urethra increases and the proportion of normal microbiota decreases compared to the structure of the normal microbiota.

At the same time, a significant increase in the quantities of different OMs (especially bacteria associated with BV and anaerobes) was detected in the composition of microbiota of STI- patients compared to normal microbiota.

TREATMENT

To prescribe the appropriate treatment it is necessary to determine and take into account the etiology of lower urogenital system disorders in men. Table 3 provides information about drugs that are recommended for the treatment of diseases of the urogenital tract caused by microorganisms detected by the Androflor® kit.

Table 4.

Parameters of the Androflor® kit	Microorganisms included in the group	Drug / group of medicines	Source of information
OMs associated with bacterial vaginosis	*Gardnerella vaginalis, Atopobium cluster, Megasphaera spp./ Veillonella spp./ Dialister spp, Sneathia spp./ Leptotrichia spp./ Fusobacterium spp.*	Nitroimidazoles: Metronidazole	[29, 30]
	Ureaplasma urealyticum, Mycoplasma hominis, Ureaplasma parvum	Doxycycline	[31, 32]
OM anaerobes	*Bacteroides spp./ Porphyromonas spp./ Prevotella spp, Anaerococcus spp, Eubacterium spp, Peptostreptococcus spp./ Parvimonas spp.*	Nitroimidazoles: Metronidazole	[33, 34, 35,]
OM *Pseudomonas aeruginosa/ Ralstonia spp./ Burkholderia spp*		Fluoroquinolones, carbapenems	[36, 37, 38]
OM *Haemophilus spp.*		Azithromycin	[39]
OM *Enterobacteriaceae/ Enterococcus spp.*		Fluoroquinolones: enoxacin, fleroxacin, lomefloxacin, ofloxacin, ciprofloxacin	[40]
Fungi *Candida spp.*		Fluconazole	[35, 40]
Pathogenes	*Chlamydia trachomatis*	Azithromycin	[40, 41]
	Mycoplasma genitalium	Azithromycin	[40, 41]
	Neisseria gonorrhoeae	Azithromycin	[40, 41]
	Trichomonas vaginalis	Nitroimidazoles: Metronidazole	[40, 41]

POSSIBLE COMPLICATIONS OF THE METHOD AND THE WAYS OF THEIR ELIMINATION

Negative side effects were not identified when using the described method for laboratory diagnostics of male lower urogenital tract diseases.

EFFICIENCY OF THE METHOD

The real-time PCR performed with Androflor®, Androflor® Screen kits allows to determine the majority of clinically significant microorganisms in patients with symptoms of lower genital tract infections. Different types of biomaterial, recommended by IUSTI, could be used for analysis.

The list of microorganisms detected by the kit was compiled taking into account the latest achievements of the Human Microbiome Project and the data on etiological role of these microorganisms in the male lower urogenital tract disorders.

Androflor® kit will allow us to significantly expand the list of detectable microorganisms the importance of which was shown during clinical trials.

Compared to culture-based methods, real-time PCR (Androflor®, Androflor® Screen kit) has a number of advantages. The requirements for collection, storage and transporting the sample are a lot less demanding. The risk of sample contamination is significantly lower. Equal sensitivity and specificity for all microorganisms (including non-culturable and difficult to culture microorganisms) is provided. And the results of the analysis are received within several hours.

CONCLUSION

Androflor® and Androflor® Screen are an effective method of laboratory diagnostics of male lower urogenital tract infections. It allows complex evaluation of the urogenital microbiota, including qualitative detection of sexually transmitted pathogens, quantitative assessment of normal microbiota, opportunistic bacteria and yeast-like fungi (*Candida spp.*). The results of the test allow the specialists to establish the causative agents of the male urogenital tract infection; work out an etiologically targeted treatment and prevent polypharmacy.

In a short time, the method allows objectively:

1) Evaluation of the qualitative and quantitative composition of the microbiota of the male lower urogenital tract;
2) Etiological diagnosis of the lower urogenital tract infections;
3) To make a decision on appropriate treatment;
4) To monitor the treatment effectiveness.

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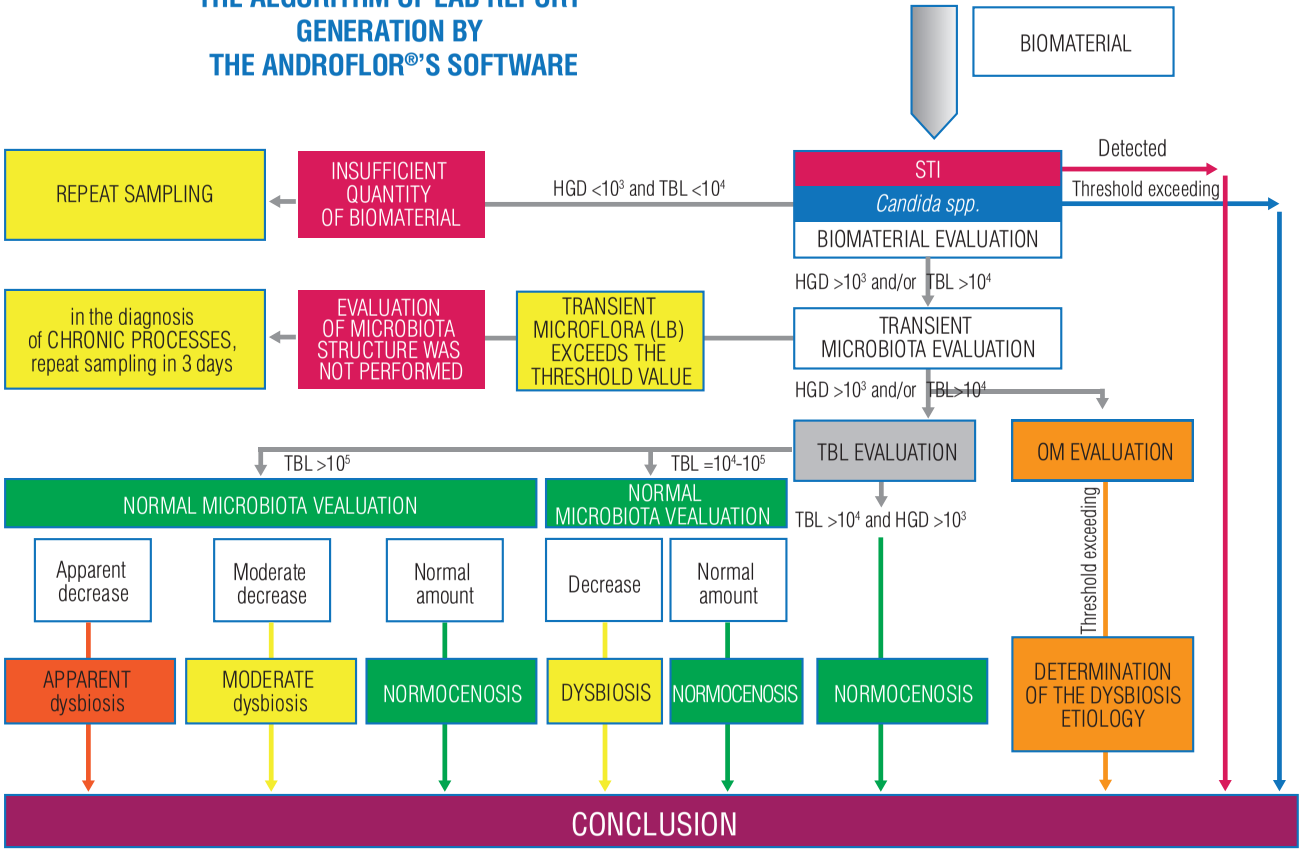
THE ALGORITHM OF LAB REPORT GENERATION BY THE ANDROFLOR®'S SOFTWARE



Abbreviations: TBL — total bacterial load, HGD — human genomic DNA, LB — Lactobacilli, OM — opportunistic microorganisms.

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