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Combination of microbiological culture and multiplex PCR increases the range of vaginal microorganisms identified in cervical cancer patients at high risk for bacterial vaginosis and vaginitis

Zastosowanie hodowli i multiplex PCR w diagnostyce drobnoustrojów wywołujących zapalenie pochwy u kobiet z rakiem szyjki macicy

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Abstract

Background: *Bacterial vaginosis (BV) and vaginitis in cervical cancer patients might becaused by mixed aerobic, anaerobic, and atypical bacteria. Since genital tract infections can be complicated, early and accurate identification of causal pathogens is vital.*

Objectives:*The purpose of this study was i) to determinate if currently used aerobic culture methods are sufficiently sensitive to identify pathogens that can appear in the cervix of women after cancer treatment; ii) to investigate if molecular methods can improve the diagnostic process of BV and vaginitis, as well as broaden the range of detectable pathogens that would otherwise be difficult to cultivate. Methods: A one-year hospital-based study was conducted in 2011/2012. Cervical swabs from 130 patients were examined by microbiological culture and multiplex PCR.*

Results: *Swab samples were positive for 107 and 93 women by microbiological culture and multiplex PCR, respectively. The most common bacteria isolated from culture were: Escherichia coli, Enterococcus faecalis, Streptococcus agalactiae, and Staphylococcus aureus, and using the molecular technique were: Gardnerella vaginalis, Bacteroides fragilis, Ureoplasma ureoliticum/parvum, Mobiluncus curtisii and Atopobium vaginae.*

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Otrzymano: **02.09.2014**
Zaakceptowano do druku: **15.11.2014**

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Conclusions: *Multiplex PCR might contribute to the diagnosis of genital tract infections and it broadens the number of detectable microorganisms responsible for BV. Combination of these two methods may become the basis for standardized diagnosis of BV and vaginitis.*

Key words: **vaginitis** / **bacterial vaginosis** / **multiplex PCR** / **microbiological culture** /

Streszczenie

Bakteryjne zapalenie pochwy u pacjentów chorych na raka szyjki macicy może być spowodowane przez bakterie beztlenowe, tlenowe i atypowe. Ponieważ infekcje narządów płciowych mogą doprowadzić do poważnych komplikacji, wczesna i odpowiednia identyfikacja źródła infekcji jest bardzo ważna.

Cel pracy: *1) określenie, czy obecnie używane metody hodowli tlenowej w pracowni mikrobiologicznej są wystarczające do identyfikacji patogenów, które mogą pojawić się w szyjce macicy po leczeniu nowotworowym; 2) zbadanie, czy molekularne metody mogą polepszyć diagnostykę bakteryjnych zapaleń pochwy i zwiększyć zakres wykrywanych patogenów o te, które są trudne w hodowli.*

Metodyka: *Materiałem do badań były wymazy z szyjki macicy, pobrane od 130 kobiet z nowotworem szyjki macicy hospitalizowanych w Wielkopolskim Centrum Onkologii w latach 2011-2012. Identyfikacji mikroorganizmów dokonano tradycyjną metodą hodowlaną i metodą molekularną- multiplex PCR.*

Wyniki: *Hodowla mikrobiologiczna zdiagnozowała 107 pozytywnych przypadków, zaś multiplex PCR- 93. Najczęściej izolowanymi patogenami metodą mikrobiologiczną były: Escherichia coli, Enterococcus faecalis, Streptococcus agalactiae i Staphylococcus aureus, a techniką molekularną: Gardnerella vaginalis, Bacteroides fragilis, Ureoplasma ureoliticum/parvum, Mobiluncus curtisii i Atopobium vaginae.*

Wnioski: *Multiplex PCR mógłby pomóc w diagnostyce infekcji narządów płciowych i powiększyć zakres wykrywanych mikroorganizmów. Połączenie tych dwóch technik może stanowić podstawę standaryzacji diagnostyki zapalenia dróg rodnych kobiet.*

Słowa kluczowe: **bakteryjne zapalenie pochwy** / **multiplex PCR** /
/ **hodowla mikrobiologiczna** /

Introduction

The normal bacterial flora of the female genital tract is composed of various aerobic and anaerobic bacteria. It is usually dominated by lactobacilli, which play an important role in preventing genital and urinary tract infections. However, healthy bacterial flora of a vagina can be replaced by undesirable bacterial pathogens that can cause conditions such as bacterial vaginosis (BV) or vaginitis among high-risk patients (after surgery, parturition or antibiotic treatment).The most common microorganisms associated with BV are *Gardnerella vaginalis, Mycoplasma* spp*., Ureoplasma ureoliticum*/*parvum* and *Mobiluncus* spp. [1, 2, 3], while the most commonly isolated microorganisms associated with vaginitis are *Candida* spp., Gram negative bacteria (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Morganella morganii*) and Gram positive cocci (*Streptococcus* spp*., Staphylococcus* spp.) [4].

Cervical cancer is animportant issue in women's health, representing the second most common malignancy, with the incidence of 500000 patients annually worldwide [5, 6]. The management of cervical cancer varies depending on the International Federation of Gynecological Oncologists (FIGO) Staging System, but radiotherapy plays a vital role across the range of presentations [7]. In patients suffering from urogenital cancer, vaginitis may be observed as a side effect of genital radiotherapy or brachytherapy, and the clinical state is often worsened by fungal or bacterial superinfection [8].

In addition, surgical procedures and radiation impair the physiological barrier of the structures and the lymphatic system causes changes of the bacterial flora [9].

BV is often asymptomatic but if symptoms are present they include vaginal irritation or malodorous discharge [10]. BV frequently recurs after antibiotic treatment, especially among immunocompromised patients, including cancer patients. The majority of women suffering from vaginal infectionsare only diagnosed using a microbiological culture, which is limited to cultivable pathogens. There is a paucity of data in the literature on the etiology of BV or vaginitis in patients with cervical cancer based on the molecular methods. In our study, we combined a molecular method such as multiplex PCR with a traditional microbiological culture to broaden the range of detectable microorganisms and provide an accurate and rapid guide for detection of pathogens responsible for BV and vaginitis. We postulated that combination of these two methods may improve the diagnosis of genital tract infections.

Material and methods

The study was conducted in the Greater Poland Cancer Centre and DNA Research Centre between 2011 and 2012.

Patient characteristics

Cervical cancers, stages IB to IVA, were diagnosed in 130 women according to the FIGO clinical classification. In all cases

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clinical findings were in agreement with histopathological diagnosis. Additionally, among these patients five other cancers were diagnosed (3 x breast cancer, 1x lung cancer, and 1x thyroid cancer). The age range was 26-86 years, with a mean of 58. Patient characteristics are presented in Table I. All patients received radical radiotherapy, which consisted of external beam radiotherapy combined with intracavitary brachytherapy, with the exception of 26 patients with stage IV cervical cancer.

None of the patients was diagnosed with BV and vaginitis prior to these procedures and there was no information about clinical symptoms of an ongoing acute inflammation of the genital tract.

Swab culture and sensitivity tests

All microbiological culture and molecular testing in this study was performed on swab specimens. Quantitative microbiological culture was performed using Chrom ID CPS chromogenic agar, D-Coccosel medium, Cetrimide agar, Albicans ID2 agar (bioMérieux, France). Cultures were prepared using quantitative loops and incubated at 35°C overnight,with the exception of Albicans ID2 agar, which was re-examined after 48h.

Microorganisms were identified according to standard biochemical tests, which identified most isolated strains to genus level and many to species level. The Vitek identification system (bioMérieux, France) was used for confirmation. *In vitro* susceptibility was determined primarily by Vitek AST GP and AST N0 systems (bioMérieux, France).

DNA isolation

DNA from swabs was isolated using NucleoSpin Tissue kit (Macherey-Nagel, Germany). One ml of the liquid covering the swab was centrifuged for 5 minutes at 13000g. Then, 180 µl of T1 buffer and 25 µl of proteinase K were added to the sediment and incubated for 1 hour at 56°C. Two hundred µl of B3 lysis buffer were added and the mixture was incubated for 10 minutes at 70°C to lyse the cells. Two hundred and ten µl of 96% ethanol was added to the mixture and then it was loaded onto the column and centrifuged for 1 minute at 11000g to bind the DNA.

To purify the DNA, 500 µl of BW buffer was passed through the membrane, followed by 600 µl of B5 buffer. The mixture was then centrifuged for 1 minute at 11000g. The DNA was eluted in 100 µl of DNase-free TE buffer (Tris-EDTA pH8), (Macherey-Nagel, Germany).

The DNA concentration was measured by NanoDrop (Thermo-Fisher Scientific, USA). The concentration of the eluted DNA was between 15-100 ng/µl.

Multiplex PCR

Multiplex PCR was performed using Seeplex STI Master ACE Detection kit (Seegene, Seoul, South Korea). This system was used to detect the following microorganisms: *Trichomonas vaginalis, Mycoplasma hominis, Mycoplasma genitalium, Chlamydia trachomatis, Neisseria gonorrhoeae, Ureoplasma ureoliticum/parvum, Gardnerella vaginalis, Bacteroides fragilis, Mobiluncus curtisii, Atopobium vaginae, Mobiluncus mulieris,* and six *Candida* species: *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, C. albicans,* and *C. dubliniensis.*

The target regions for bacterial genes were not specified by the multiplex PCR producers.

The PCR reaction was prepared according to the manufacturer's instruction. The PCR solution contained DNA polymerase, dNTP, $MgCl_2$, DNA internal control, primers to internal control and pairs of specific primers to DNA microorganism. The PCR reaction was amplified in a DNA Engine® thermal cycler (Bio-Rad, USA) during 40 thermocycles consisting of a 30-s denaturation step at 94°C, a 90-s annealing step at 63°C and 90-s elongation step at 72°C. Positive, negative and internal controls were used in each PCR reaction. Amplicons were visualized under UV light after electrophoresis in 2% agarose gel containing GelRed (Biotium). Fragment sizes were determined by a comparison with a DNA size marker STI1, STI2 and STI3 DNA ladder (Seegene, South Korea) (Figure 1).

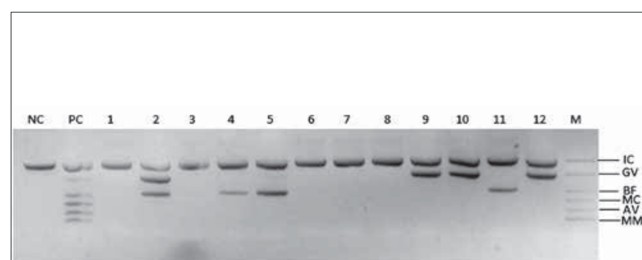

Figure 1. Agarose gel electrophoresis of multiplex PCR amplifield products generated from patient DNA samples using Seeplex STI Master ACE Detection kit. Lane NC – negative control; lane PC – positive control; Lane M – STI DNA ladder; lanes 1,3,6,7,8 – negative samples; lane 2 positive sample (*Gardnerella vaginalis, Bacteroides fragilis*); lanes 4,5 – positive samples (*Bacteroides fragilis*); lanes 9,10 – positive samples (*Gardnerella vaginalis*); lane 11 – positive sample (*Bacteroides fragilis*); lane 12 – positive sample (*Gardnerella vaginalis*). IC – internal control (981bp Plasmid DNA); GV – (*G. vaginalis*); (661bp); BF – *B. fragilis (415bp); MC – M. curtisii* (320bp); AV – *A. vaginae* (240bp); MM – *M. mulieris* (182bp).

Results

PCR and culture results

Swab samples were positive for 107 and 93 women by microbiological culture and multiplex PCR, respectively. For both techniques, positive and negative results were observed in 74 and 4 cases, respectively. A total of 33 patients had positive results by microbiological culture only, while 19 had positive results by PCR only. We observed that 28 women had monomicrobial infections, while polymicrobial infections were identified in 46 cases. Infection caused by more than two microorganisms was observed in 10 patients when combining the results from both methods (Table II). The number of microorganisms detected by culture and multiplex PCR is shown in Table III. The most frequently isolated microorganism by microbiological culture was *Escherichia coli* with 43 (26%) cases identified, followed by *Enterococcus faecalis* (26, 15.5%), *Streptococcus agalactiae* (20, 12%) and *Staphylococcus aureus* (14, 8.5%). The most common bacteria detected by multiplex PCR were *Gardnerella vaginalis* (49, 26%) and *Bacteroides fragilis* (42, 22%), followed by *Ureoplasma ureoliticum/parvum* (26, 14%), *Mobiluncus curtisii* (23, 12.5%) and *Atopobium vaginae* (21, 11%). Eight patients were positive for parasitic *Trichomonas vaginalis* infection. *Mycoplasma hominis* was detected in 5 cases, while only one case of *Mycoplasma genitalium* and *Neisseria gonorrhoeae*

was identified. PCR test was negative for all *Candida* spp. included to assay (Table III). Microorganisms detected by culture and PCR were not confirmed by other techniques.

Discussion

Studies in the last decade have established that bacterial vaginosis can be associated with various complications (e.g. post-surgical sepsis, vulvovaginitis, pelvic inflammatory disease, endocervicitis), and may play a role in cervical carcinogenesis [11, 12, 13]. Therefore, accurate and rapid detection of microorganisms causing genital infections is essential to treat and prevent further complications, particularly among immunocompromised patients. In the present work we combined the gold standard microbiological culture with a molecular method to find out whether molecular technique (multiplex PCR) can detect additional, difficult to cultivate, pathogens in the vaginal flora which traditional cultures did not identify, in a group of patients after cancer treatment. In our study, such combination of the techniques gave the correct outcome in 93 out of 130 cases (71.5%), while the remaining 33 samples were only positive by the conventional method, and 4 samples were negative by both methods. Thus, we postulate that the combination of methods may improve the diagnostic process of genital tract infections and help in selecting proper antimicrobial therapy at an early stage.

BV is mainly caused by anaerobic and atypical bacteria, whereas aerobic microorganisms are responsible for vaginitis. Cancer patients after cervical treatment are in the high-risk group for the development of BV and vaginitis. Both infections are caused by an increase in pH (>4.5), which is conducive to growing undesirable pathogens. Changes in the vaginal pH may arrest squamous metaplasia in the post-pubertal cervix and prolong the period in which the transformation zone is vulnerable to agents promoting dysplasia such as HPV [14]. Moreover, side effects after cancer treatment, i.e. soft tissue damage after external radiotherapy or brachytherapy, cause serious effects and may have a negative influence on patient immune system [15]. The high proportion of patients with anemia (23.9% of our cohort) should be noted. It is specific for those with advanced cancer [16, 17], including cervical cancer [18].

The diagnosis of BVand vaginitis has been largely dependent on methods such as culture, enzyme immunoassay, DNA hybridization, gas-liquid chromatography, antibody staining, and microscopic analysis [19, 20]. A Gram strain and microbiological culture are the gold standard for the diagnosis of genital infections in many hospital laboratories. However, these methods provide very limited information regarding identities and relative abundance of the organisms present in a sample [11]. Although in recent years the use of molecular methods has become more common in clinical samples, they are still rarely used in identifying pathogens causing BV and vaginitis. In our study, we broadened the screening test not only for bacteria that are difficult to cultivate but for anaerobic microorganisms and parasites as well. We found that atypical and anaerobic pathogens occur in the same frequency as aerobic ones among cervical cancer patients. Among 130 women, we identified 166 strains by culture and 18 additional microorganisms by multiplex PCR.

In order to improve the diagnosis of genital tract infections we developed a method that combines the results from routine aerobic culture with those from a commercially available multiplex PCR assays (SeeplexSTI Master ACE Detection kit, Seegene, Seoul, Korea), designed to detect the most common pathogens associated with genital tract infections. This method broadens the range of pathogens detectable by either method alone and has the potential to significantly improve the prognosis/diagnosis of genital tract infections, especially in high-risk patients such as those suffering from cervical cancer. Horii *et. al.* [21], were the first to describe the application of the Seegene system in detecting six

Table I. Patient characteristics.

Number of patients	130
Ages	26-86 (56) years
Stage of uterine cervix cancer	**Number of patients**
IB	2
IIB	41
IIIB	61
IV	26
Additional clinical information	**Number of patients**
arterial hypertension	31
anaemia	31
diabetes	14
urinary bladder infiltration	13
metastases to: vagina, lymph nodes, lung	10
hydronephrosis	8
urinary bladder extraction	5
sciatic nerve, vagina, vulva infiltration	4
renal failure	4
thyroid, vermiform appendix extraction	3
obesity	3
uraemia	3
vaginal extraction	2
duodenal ulcer	2
thrombosis	2

Table II. Number of infected patients detected by microbiological culture and multiplex PCR.

Culture	**PCR**	**Result of patients**
Negative	Negative	4
Positive	Positive	74
Positive	Negative	33
Negative	Positive	19
Total		**130**
Monomicrobial	Monomicrobial	28
Infection ≥2 pathogens	Infection ≥2 pathogens	10
Monomicrobial	Infection ≥2 pathogens	14
Infection ≥2 pathogens	Monomicrobial	22
Total		**74**

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Table III. Number of microorganisms from swab samples detected by microbiological culture and multiplex PCR.

Nr	Microbiological culture	Result	Multiplex PCR	Result
1	*Escherichia coli*	43	*Gardnerella vaginalis*	49
2	*Enterococcus faecalis*	26	*Bacteroides fragilis*	42
3	*Streptococcus agalactiae*	20	*Ureoplasma ureoliticum/parvum*	26
4	*Staphylococcus aureus*	14	*Mobiluncus curtisii*	23
5	*Streptococcus anginosus*	6	*Atopobium vaginae*	21
6	*Streptococcus sanguis*	5	*Trichomonas vaginalis*	8
7	*Staphylococcus haemolyticus*	5	*Mobiluncus mulieris*	6
8	*Streptococcus group C*	4	*Mycoplasma hominis*	5
9	*Streptococcus gallolyticus*	4	*Chlamydia trachomatis*	2
10	*Streptococcus mitis*	4	*Mycoplasma genitalium*	1
11	*Staphylococcus epidermidis*	4	*Neisseria gonorrhoeae*	1
12	*Micrococcus* spp.	4	*Candida glabrata*	0
13	*Streptococcus* spp.	3	*Candida tropicalis*	0
14	*Enterobacter cloacae*	3	*Candida parapsilosis*	0
15	*Morganellamorganii*	3	*Candida krusei*	0
16	*Proteus mirabilis*	3	*Candida albicans*	0
17	*Streptococcus group A*	2	*Candida dubliniensis*	0
18	*Streptococcus group G*	2		
19	*Staphylococcus warneri*	2		
20	*Klebsiella pneumoniae*	2		
21	*Streptococcus group F*	1		
22	*Streptococcus bovis*	1		
23	*Streptococcus parasanguis*	1		
24	*Streptococcus pyogenes*	1		
25	*Enterococcus avium*	1		
26	*Raoutella planticola*	1		
27	*Candida parapsilosis*	1		
Total		**166**		**184**

sexually transmitted pathogens, namely *Chlamydia trachomatis, Neisseria gonorrhoeae, Ureoplasma urealyticum, Mycoplasma genitalium, Mycoplasma hominis* and *Trichomonas vaginalis* in clinical specimens. In our study, we used a more recent version of the productcapable of detecting 17 microorganisms in total, including those causing sexually transmitted infections (STIs), bacterial vaginosis, and fungal pathogens. We believe that this method will be more time- and cost-efficient than other currently used techniques, due to the high number of organisms it can detect.

Gynecological diseases affect the growth of vaginal bacterial flora, especially anaerobic bacteria, such as *Gardnerella vaginalis* and *Bacteroides fragilis* [22, 23].

In our research, these microorganisms were the most frequently identified, detected in 49 (26%), and 42 (22%) cases, respectively. The presence of these pathogens and additional *Mobiluncus curtisii* in the genital flora are found in subjects who do not have bacterial vaginosis, therefore they are not specific markers for BV [1]. In our work, we did not have the negative control group because the aim of our investigation was to find out which other microorganisms, except aerobic bacteria, can occur in the vaginal flora of high-risk patients. Moreover, it was reported [22, 23] that anaerobic strains become predominant in cervical cancer patients and this alteration may be related to the malignancy itself or by management protocol such as radiation therapy [9]. In some cases those pathogens can lead to the development of septic arthritis of the hip joint [9], or increase the risk of getting pelvic inflammatory disease (PID), especially after surgical procedure like hysterectomy, or other STIs caused by *Chlamydia trachomatis*, which is the most commonly observed etiological factor of STI, or *Neisseiria gonorhoeae* [24, 25].

The second most frequent pathogen detected by the molecular technique was *Ureoplasma ureoliticum/parvum.* This bacterium appears in the cervix or the vagina in 40-80% of the asymptomatic women. Additionally, there is growing evidence that it is associated with a range of human disorders, including respiratory infection, pelvic inflammatory disease, intrauterine infection, and non-gonococcal urethritis [3, 26, 27, 28]. Stellrecht *et al*. [3], reported that multiplex PCR is more sensitive than culture in the detection of *Ureoplasma urealiticum* and *Mycoplasma* spp. In their research, PCR enhanced the detection rate of genital mycoplasma to 24%, and the presence of other pathogens did not interfere with testing.

Mobiluncus curtisii, Mobiluncus mulieris and *Atopobium vaginae* are anaerobic bacteria which, similarly to previous aerobic pathogens,could be easily detected by anaerobic culture. Among our patients we did not cultivate those microorganisms anaerobically. However, we detected them additionally by multiplex PCR (Table III). Although they could be interpreted as normal microflora in the vagina, in some cases they can lead to tubo-ovarian abscess, uterine endometritis, and sepsis [29, 30].

In our results we discovered some cases of atypical bacteria and parasites (*Mycoplasma hominis* – 5, *Mycoplasma genitalium* – 1, *Chlamydia trachomatis* – 2 and *Trichomonas vaginalis* – 8).

Those pathogens are very rarely diagnosed in routine examination because they are fastidious, culture is time-consuming, and they need a specialized medium. However, identification of those pathogens could be crucial for immunocompromised patients. *Trichomonas vaginalis*is sometimes diagnosed during microscopic analysis of urine. It has been estimated that between 10% and 50% of *Trichomonas vaginalis* infections are asymptomatic [31, 32]. Moreover, this parasite has also been implicated as a cofactor in the transmission of the human immunodeficiency virus and other STD agents. A relationship between *Trichomonas vaginalis* and cervical cancer has been suggested [32].

E. coli was the most frequently detected by culture (26%) among aerobic bacteria. Although this pathogen is not generally regarded as the specific cause of BV, it is claimed to be associated with aerobic BV [10]. *Enterococcus faecalis* (16%), *Streptococcus agalactiae* (12%) and *Staphylococcus aureus* (8.4%) were also one of the factors responsible for vaginal disorder and, similarly to other gram positive and gram negative bacteria, they can occur in the periods of resistance reduction. *Staphylococcus epidermidis* was detected in four samples. Although this skin commensal is not normally pathogenic, it can take advantage of immunodeficiency or abnormalities in the urogenital tract and can cause urinary tract infections [10].

In our research, more than half of the patients (62%) were diagnosed with polymicrobial infections. They presented not with only aerobic infections, but anaerobic and atypical infections at the same time. In routine laboratory diagnostic tests, these patients were only diagnosed via the microbiological aerobic culture. It has been suggested they were misdiagnosed and they have received inappropriate and insufficient treatment. Identification of additional atypical and anaerobic pathogens could be helpful in recovery for those groups of patients and prevent serious further complications.

Although we demonstrated that multiplex PCR identified additional pathogens that are difficult to detect using the traditional culture, it will never replace it. The weaknesses of the molecular methods are the following: limitation of detecting certain pathogens, the cost of equipment, and availability of commercial kits. In combination with microbiological culture, both techniques compose a perfect diagnostic tool to identify BV and vaginitis, which might significantly help in selecting proper antimicrobial therapy at an early stage. Culture can easily identify aerobic bacteria and PCR can detect pathogens that are incapable of growing into colonies. Such combination of techniques could create a guideline for rapid and sensitive diagnosis of BV and vaginitis in urgent cases. However, it should be emphasized that when the lines are blurred between commensal and pathogen detection, the clinical symptoms of patients are paramount. Some infections caused by atypical bacteria can be asymptomatic, but their identification may be crucial, especially in immunocompromised patients, to prevent further morbidity and mortality. Additionally, the absence of clinical symptoms of an ongoing acute inflammation in the genital tract does not rule out the possibility of such etiological agents causing inflammation being present in small populations and keeping the inflammatory process of the genital tract around the sub-threshold level [33]. Therefore, we believe that molecular methods, such as multiplex PCR assay, can supplement microbiological culture when identifying fastidious microorganism in urgent cases and may be helpful in choosing effective treatment. If BV can be better diagnosed, its link to the development of cervical cancer can be better understood and may lead to the introduction of more frequent screening of women with a history of recurrent or persistent BV.

Conclusions

The result of our study indicate that combination of microbiological culture and multiplex PCR increases the range of vaginal microorganisms identified in cervical cancer patients. Using only aerobic microbiological culture is insufficient to diagnose an inflammation of the vagina in this group of patients. In the future, it is necessary to implement a reliable broad-range detection system for routine diagnosis of BV and vaginitis,which covers a wide range of clinically relevant microbes and carefully evaluates the diagnostic potential of these methods.

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7. Joanna Odważna – wykonanie i analiza badań laboratoryjnych.
8. Jacek Wojciechowicz – współautor koncepcji i założeń pracy, akceptacja i weryfikacja manuskryptu.

Źródło finansowania:

Praca nie była finansowana przez żadną instytucję naukowo-badawczą, stowarzyszenie ani inny podmiot, autorzy nie otrzymali żadnego grantu.

Konflikt interesów:

Autorzy nie zgłaszają konfliktu interesów oraz nie otrzymali żadnego wynagrodzenia związanego z powstawaniem pracy.

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