ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/305768976

Detection and phylogenetic characterization of Gardnerella vaginalis vaginolysin in samples from Bulgarian women w....

Article *in* Comptes rendus de l'Académie bulgare des sciences: sciences mathématiques et naturelles · July 2016

CITATION
1

READS
107

4 authors, including:

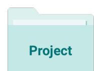
Ivo Sirakov
Medical University of Sofia
46 PUBLICATIONS 26 CITATIONS

SEE PROFILE

Tanya Strateva
Medical University of Sofia
63 PUBLICATIONS 702 CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Bacterial fish diseases in Bulgaran aquaculture View project

All content following this page was uploaded by Ivo Sirakov on 02 August 2016.

The user has requested enhancement of the downloaded file.

Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences
Tome 69, No 8, 2016

MEDECINE
Microbiologie

DETECTION AND PHYLOGENETIC CHARACTERIZATION OF *GARDNERELLA VAGINALIS* VAGINOLYSIN IN SAMPLES FROM BULGARIAN WOMEN WITH BACTERIAL VAGINOSIS

Raina Gergova, Ivo Sirakov, Ivan Mitov, Tanya Strateva

(*Submitted on February 22, 2016*)

Abstract

Recently, the intensive use of more advanced methods, including molecular genetic analysis, has aided the studies on the etiological role of *Gardnerella vaginalis* as the major pathogen of bacterial vaginosis (BV). The objective of our study was to propose a polymerase chain reaction (PCR)-based method detecting the *G. vaginalis* vaginolysin gene (*vly*) and to perform a phylogenetic analysis in an attempt to find possible correlations between clinical manifestations and gene mutations. Vaginal samples were collected from 1145 women with symptoms for vaginitis and 378 asymptomatic ones of reproductive age. After PCR detection of *G. vaginalis* 289 DNA samples were tested for *vly* using primers targeted at amplification of the whole gene. The presence of *vly* was detected in all 289 samples with positive PCR for *G. vaginalis*. Sixteen *vly*-positive PCR products were sequenced. The phylogenetic analysis based on the reference nucleic acid sequences deposited in the NCBI GenBank, covering the whole *G. vaginalis vly* gene, placed the studied isolates into three main groups. In conclusion, our experiments confirmed the major *G. vaginalis* virulence factor to be vaginolysin. The multiplex PCR results are promising and further studies should be carried out to determine the sensitivity range of the method and its applicability to diagnostics. Our phylogenetic analysis results, where some samples were grouped together, might imply a possible correlation between the clinical signs, the tendency to chronification, the cytotoxicity of vaginolysin and the *vly* gene mutations.

Key words: *Gardnerella vaginalis*, vaginolysin, phylogenetic analysis

Introduction. *Gardnerella vaginalis* is a small, non-motile, Gram-variable coccobacillus that can grow in microaerophilic conditions. In the years following

This study was supported by a grant from the Medical University of Sofia, (Council of Medical Science, Project No 37/2013, Grant No 18/2013).

the discovery taxonomic status of this microbe remained uncertain due to some specifics in its cell wall structure: the cell wall stains Gram-positively in the exponential growth phase but, as the culture ages, the peptidoglycan layer becomes thinner and gives Gram-negative staining [1]. Thus, this Gram-variable microorganism was initially classified in the *Corynebacterium* and *Haemophilus* genera, but was later moved to a separate genus under the name *G. vaginalis* [1]. This bacterial species was proved to be a causative agent of genital tract infections in studies with volunteers [2] and in animal models [3]. It was also detected in exfoliated vaginal epithelium, in the absence of an inflammatory infiltrate. In recent years, the intensive use of more advanced methods, including molecular genetic analysis, has aided the studies on the etiological role of *G. vaginalis*, identifying it to be the major pathogen (in terms of frequency and quantity) in women with bacterial vaginosis (BV) [4–6]. Several members of the genus *Lactobacillus* inhibit pathogenic bacteria in healthy women‘s vaginal ecosystem but some substances produced by *G. vaginalis* could provide a synergistic effect for the growth of the other pathogens as well as an inhibitory effect against lactobacilli [3]. BV is the most frequent vaginal infection among women in the reproductive age group, and surveys on different continents alarm that it is especially frequent in younger women, under 30 years of age [5,7].

Several attempts have been made to classify *G. vaginalis* strains based on the phenotype; however, this proved to be clinically irrelevant [8]. The major *G. vaginalis* virulence factor is an exotoxin with cytotoxic activity (including hemolytic activity) against human cells. The hemolysin triggers a local immune response and the synthesis of secretary immunoglobulin A (sIgA) can therefore be used as a diagnostic marker for BV. This toxic product is a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins [9]. The *G. vaginalis* hemolysin, termed vaginolysin (VLY), shows selective toxicity and specifically targets host cells that carry complement regulatory molecule CD59. The cytotoxicity mechanism is based on VLY-mediated pore formation, with the proline residue in the undecapeptide of domain 4 of the toxin molecule playing the main role. Mutations in this region generate a VLY toxoid and could possibly aid in the development of a vaccine [9].

The aim of the present study was to propose a polymerase chain reaction (PCR)-based method for detection of the *G. vaginalis vly* gene and to perform a phylogenetic analysis in an attempt to find possible correlations between some clinical manifestations and certain gene mutations.

Materials and methods. ***Light microscopy analysis.*** The Nugent score [10] from 0 to 10 was used to classify direct Gram-stained smears from 1523 examined female patients (1145 women with evident clinical symptoms of vaginal discharge and from 378 asymptomatic women (without vaginal discharge) of reproductive age – range 16–45 years into three groups for convenience. Grade I (Nugent score of 0–3) was considered normal vaginal microflora (NF); grade II

(Nugent score of 4–6) was intermediate between normal microflora and bacterial vaginosis (IF), and grade III (Nugent score of 7–10) was considered typical bacterial vaginosis (BV).

Swab cultures. Vaginal swabs were collected in transport medium by a gynaecologist specialist parallel to the direct smears. Samples were screened as previously described [5].

Samples and DNA extraction. DNA was isolated from the vaginal swabs with the DNAsorb-AM nucleic acid extraction kit (AmpliSens), according to the manufacturer's instructions. DNA obtained from pure cultures of *G. vaginalis* ATCC 14018 and *G. vaginalis* ATCC 49145 was used as a control. A total number of 289 samples were tested for vaginolysin: 243 vaginal swabs from women with clinical signs of genital infection, microscopically proven BV based on the Nugent score and *G. vaginalis*-positive PCR results; 16 samples from clinically healthy women of which were microscopically diagnosed with NF and *G. vaginalis*-negative PCR results, another 28 with IF based on the Nugent score again and *G. vaginalis*-negative PCR results, and two, with asymptomatic *G. vaginalis* BV confirmed by PCR.

Primer design and PCR. To amplify the whole 1638 bp length of the *vly* gene of *G. vaginalis*, the primers described by Gelber et al. [9] were revised in line with any new sequences deposited in GenBank, NCBI (National Center for Biotechnology Information): HQ593656; HQ593658; HQ593662; HQ593664, HQ593667, EU522486.1, EU522487.1 and EU522488.1. The primers used are listed in Table 1. PCR was carried out in 25 µL volumes (3.0 µL of DNA, 10 pmol of each primer, 7.5 µL of dH_2O, 12.5 µL of MyFy MasterMix, Bioline, UK) in a Thermocycler QB – 96 (LKB, Bulgaria). The following settings were used: Heated Lid 110 °C ; Hot Start 95 °C – 3 min., Start Cycles 30 – Denaturation 95 °C – 30 s, Gradient 50 – 66.3 °C – 30 s, Elongation 72 °C – 45 s and Final Elongation 72 °C – 7 min. Primers were analyzed for close homology, using the Basic Local Alignment Search Tool (BLAST) tool available at the NCBI database (Bethesda, MD) (http//:www.nbi.nlm.nih.gov/BLAST). MUSCLE [11] and MEGA version 5 software [12] was used for multiple alignments of nucleotide reference sequencing and primers design. Internal fragments of the *vly* gene of *G. vaginalis* were amplified with the primer pairs F1-GVaglysRLong and F1-GVaglysRshort. Gradient PCR was carried out in 25 µL volumes with total DNA from clinical samples (3.0 µL of DNA, 10 pmol of each primer, 7.5 µL of dH_2O, 12.5 µL MyTaq PCR mix, Bioline, UK) and with the amplified DNA of the whole *vly* gene (1.0 µL of DNA from PCR, 10 pmol of each primer, 9.5 µL of dH_2O, 12.5 µL MyTaq PCR mix, Bioline, UK). The PCR reactions were performed in a Thermocycler QB – 96 (LKB, Bulgaria) with the following settings: Heated Lid 110 °C, Hot Start 95 °C – 5 min, Start Cycles 35 – Denaturation 95 °C – 30 s, Gradient 50 – 66.3 °C – 45 s, Elongation 72 °C – 45 s and Final Elongation 72 °C – 7 min. After optimization of the PCR reactions for the internal fragments, Multiplex

PCR was used for their detection with PCR HS Mix (Bioline, UK) in 50 µL reaction volumes. The quality and quantity of the extracted DNA samples and of the PCR products was assessed by means of GeneQuant II RNA/DNA Calculators (Pharmacia Biotech, UK) and 2% agarose gel electrophoresis (Bioline, UK): 10 ng/mL of ethidium bromide (Sigma, USA), 1× Tris-borate-EDTA (TBE) buffer, DNA marker HyperLadder™50 bp (Bioline, UK), 120 V, 70 mA, 12 W for 40 min.

Sequencing and phylogenetic analysis. Selected vly PCR products (n = 16) were chosen for DNA sequencing. They were obtained from women with BV, of different age groups and with different clinical symptoms. All these sixteen DNA samples had been confirmed to have vaginolysin-producing *G. vaginalis* as the etiological agent of BV. A brief description of the samples follows: Samples 6, 12, 15, 79 were collected from pregnant women. The other ones were from non-pregnant. Samples 12 and 79 were obtained from 38- and 23-year-old asymptomatic (with no presence of vaginal discharge) patient, respectively. The direct Gram-stained smear and PCR assay indicate *G. vaginalis*-associated BV. The origin of samples 15, 19, 42, 84, 89 was from 27-, 19-, 25-, 32- and 36-year-old patient, respectively, with clinical symptoms and microbiological diagnosis of typical BV. Samples 53 and 181 – women, resp. 28 and 40-year-old, with clinical diagnosis of complicated vaginitis combined with cervical erosion. There were microbiological data for infection, classified by direct Gram-stained smear such as BV plus Trichomonas vaginalis. Samples 6 and 41 were collected from 25- and 27-year-old persons with chronic recurrent BV (relapse of vaginitis after therapy). The last microbiological results showed BV and initial candidiasis (isolates of Candida albicans 10^4cfu/mL in the both cases). Samples 39, 100, 137, 138, 145,

Table 1

Oligonucleotide primers complementary to the *vly* gene of *Gardnerella vaginalis*. Primer F1 begins before the start codon and the positions were determined based on reference sequence HQ593658

Primer name/ pairs	Sequence	Position	Size of product	Source
$F1$	5′-A KSCAGCGAAGCATGCCATGC-3′	4–24		Gelber et al., [9][x]
$R1$	5′-TYAGTCGTTCTTTACAGTTTC-3′[x]	1641–1621		
$GVaglysRLong$	5′-AAGCCGTTCACTGCGGAAGT-3′	614–595		this study
$GVaglysRshort$[xx]	5′-GTTCTCAATGGTTTCGCCAT-3′	315–296	1638 bp	this study

[x]Modified in this study in line with up-to-date GenBank data differences from the original primers are underlined

[xx]specific for phylogenetic group 1

were from women (35, 29, 30, 33, 35-year-old) with chronic recurrent BV, some of them after precedent therapy. The microbiological and molecular genetic techniques detected only *G. vaginalis* (without candidosis). The sixteen DNA samples described above were amplified by PCR, consecutively with primer pairs F1-R1, F1-GVaglysRLong and F1-GVaglysRshort. The sixteen clinical samples, which gave positive amplification results with the three primer pairs, were sequenced with the F1-GvaglysRLong primer pair by using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Giles, UK) and MegaBACE 1000 (Amersham Biosciences) as follows. The PCR products were purified through S400 columns (GE Healthcare, Giles, UK). The sequencing PCR mix contained: 5 pmol/mL of primer, 3.5 µL of DNA, 8.0 µL of DYEnamic ET Dye Terminator premix and dH_2O to 20 µL. The following program was used: 25 cycles at 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. Two controls were included: a control for the reaction and a control for the detection of sequencing. The phylogenetic analysis included the sequenced PCR products of the vly gene fragment between primers F1-GVaglysRLong in the five clinical samples and the relevant reference sequences in GenBank, NCBI. Multiple alignment was done with MUSCLE [11] by MEGA version 5 software [12] J Model Test 0.1.1 [13] was used to find the best model for construction of phylogenetic trees based on nucleotide acids. Prot Test 2.4 [14] was used to find the best model for the construction of phylogenetic trees based on amino acid sequences. PHYML 3.0 by Phylemon 2 [15] and 1000 bootstrap replications were used to build a phylogenetic tree. For graphical representation of the phylogenetic trees FigTree1.4.0 software was used (`http://tree.bio.ed.ac.uk/`). Selected sequences have been submitted to the GenBank, NCBI, with accession numbers as follows: KM221028, KM221029, KM221030, and KM221032.

We have informed consent signed by every examined patient. There was no personal information in the database. The patients were not included in medicine trails or any experimental therapy. The therapy prescribed was as all the clinical practice and guidelines approved, according to the current pathogen.

Results. BLAST analysis of the primers provided an identity value of 100% with the vly gene of *G. vaginalis*. The presence of the vly gene was detected in all the tested samples with positive PCR for *G. vaginalis*. The results from the phylogenetic analysis based on the reference nucleic acid sequences deposited in GenBank, NCBI, covering the whole vly gene of *G. vaginalis*, placed the studied isolates into three main groups (Fig. 1). The first group was the smallest one – it consisted of only two Bulgarian vly sequences (12 and 79) from patients with asymptomatic BV. The second group (samples 5, 19, 84, 89, 53, 181) was more heterogenic – from women with typical BV with or without T. vaginalis co-infection and complications. The last group (6, 39, 41, 42, 100, 137, 138, 145) consisted of patients with recurrent BV. The same topology was obtained with the amino-acid phylogenetic analysis.

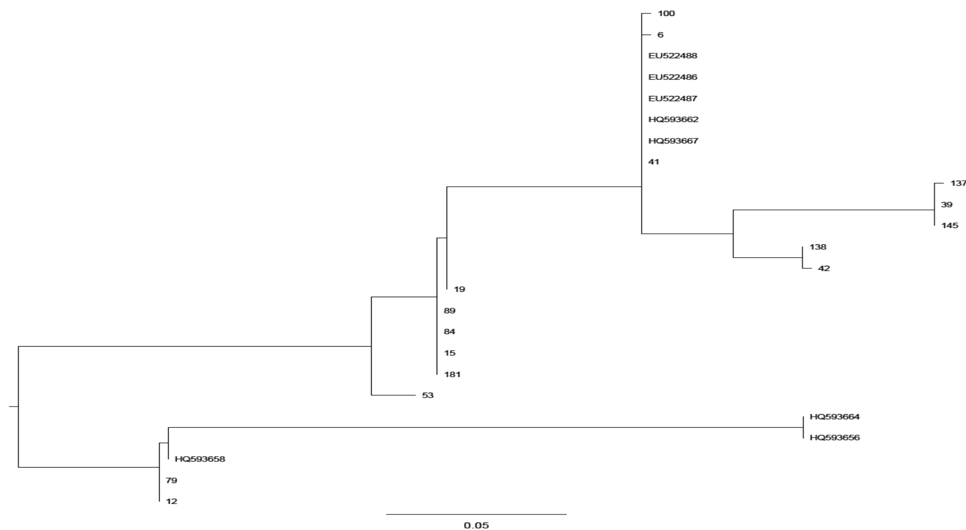


Fig. 1. Phylogenetic tree of the *vly* gene sequences of *Gardnerela vaginalis*. The tree was constructed based on the whole gene nucleotide length, aligned by MUSCLE. TIM3+I model, 1000 bootstrap replications and PHYML 3.0 were used to build the phylogenetic tree

Discussion. The amplification of the whole *vly* gene of a reference *G. vaginalis* strain with gradient PCR and F1-R1 primers, produced a 1638 bp fragment at annealing temperatures in the range of 51.4 – 61.1 °C, with optimum results at 55.8 °C (Fig. 2). When the PCR reaction was performed with the F1-GvaglysRLong primers on the whole *vly* from the first amplification reaction and on the reference strain DNA, the optimum annealing temperature for amplification of a 611 bp fragment from the *vly* was 57.7 °C. A specific but weaker 611 bp band was obtained in a temperature range of 53–63.5 °C (Fig. 2). To differentiate between the phylogenetic groups, we constructed the GvaglysRshort primer, which is specific for group 3 and in combination with the F1 primer covers a 312 bp fragment. Using gradient PCR, a specific product was amplified at all the annealing temperatures tested from 50 °C to 66.3 °C (Fig. 2). The annealing temperature that was chosen for our subsequent experiments was 57.7 °C, in accordance with the PCR results with the first internal primer. After optimizing the reaction settings for amplification of internal fragments of the *vly* gene, we carried out multiplex PCR to confirm the results (Fig. 3). The multiple sequence alignment of sixteen positive samples showed a 522 bp fragment to be suitable for analysis. Development of an efficient and cost effective PCR protocol depends on the reaction components (Taq DNA polymerase, buffers, deoxynucleoside triphosphates (dNTPs), $MgCl_2$concentration, DNA template and oligonucleotide primers), which need to be adjusted with precision. The components that are most important for the sensitivity and effectiveness of the amplification reaction are the primers and Taq DNA polymerase as well as the manufacturer in

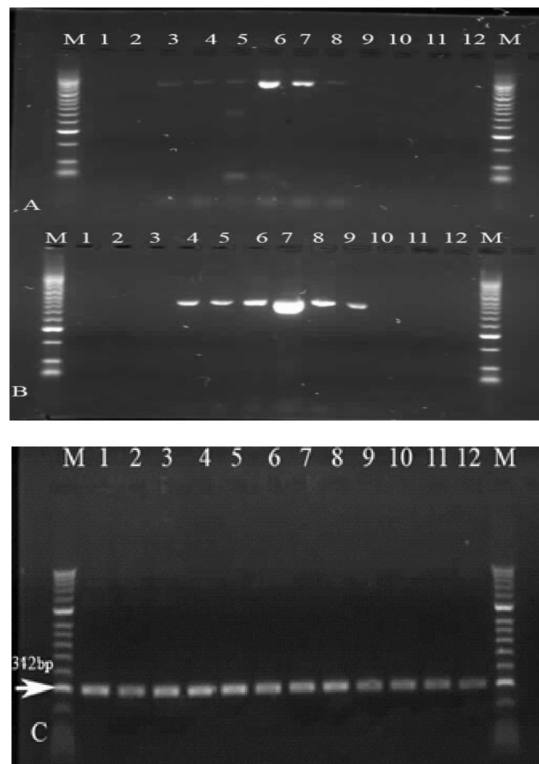


Fig. 2. Electrophoretic separation of gradient PCR products of *G. vaginalis* ATCC 14018: Annealing temperatures in the 50.0 °C – 66.3 °C range. M – DNA ladder; (*A*) amplification of the whole 1638 bp vly gene (*B*) a fragment (611 bp) between the F1-GvaglysRLong primers; (*C*) a 312 bp fragment between the F1-GVaglysRshort primers

view of different formulations, assay conditions and/or unit definitions [16]. That is why, in our experiments we chose ready-to-use PCR reaction mixes suited for amplification of fragments of the expected length. In order for a primer to be stable, its $3'$ end should be unstable and its $5'$ end, stable, which can be achieved by choosing G or C to be present in the last five $5'$ bases [17] and by complementarity. For the purpose of our study we modified two bases in the forward primer described by other authors [9] and one base at the $5'$ end of the reverse primer, in line with the new data available in GenBank. The result was that the reaction was improved in terms of higher specificity and a wider range of detected *vly* gene variants in *G. vaginalis*, thus allowing us to optimize the proposed PCR protocol [9]. Using primers targeted at amplification of the whole *vly* gene, our experiments confirmed the major *G. vaginalis* virulence factor to be vaginolysin in all the clinical samples with BV diagnosis. These results were confirmed with the F1-GvaglysRLong primer pair, proving their specificity and sensitivity. The PCR products obtained at all the annealing temperatures tested with the F1-

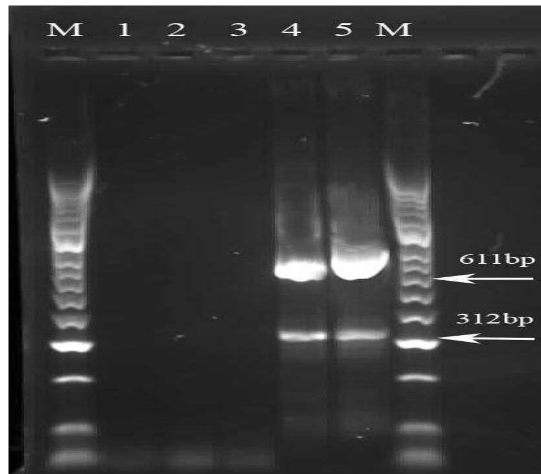


Fig. 3. Electrophoretic separation of multiplex PCR products amplified with two primer pairs: F1-GvaglysRLong (611 bp) and F1-GvaglysRshort (312 bp). M – DNA ladder; 1 – negative control with dH_2O; 2 – negative control (sample from a healthy woman); 3 – negative control (sample from a patient with microbiological diagnosis other than BV – candidosis); 4 – sample from a woman with clinical signs and microscopic diagnosis of BV; 5 – positive control (ATCC 14018: *G. vaginalis*)

GVaglysRShort primers could most probably be attributed to the presence of GC at the 3′ end, leading to stability and altered kinetics, which renders them inapplicable at a wider annealing temperature range. The multiplex PCR results are promising and further studies should be carried out to determine the sensitivity range of the method and its applicability to diagnostics. Our phylogenetic analysis results demonstrated a distribution of the samples into three major groups. The samples in the first group (with low virulent and probable slight adhesive *G. vaginalis*) were from asymptomatic BV. These sequences were very similar to the known *vly* sequence (GenBank accession no. HQ593658). Four samples – 5, 19, 84, 89 in the second group were obtained from patients with typical first time detected BV. Samples 53 and 181 from the second group, too were collected from BV-positive women with *T. vaginalis* co-infection. There are no analogical to this heterogenic group sequences deposited in the NCBI GenBank. The *vly* sequences 6, 41 and 100 (patients with recurrent BV) were 100% identical to five sequences deposited in the NCBI GenBank and formed the first subgroup of the third major group. The second (137, 145 and 39) and the third subgroup (42 and 138) included samples from women with prolonged severe infection. Here, there was a possible correlation between the most expressed clinical signs of BV, the tendency to develop a long-term (chronic) infection, the cytotoxicity of vaginolysin, the mutations in the *vly* gene, and the effect of adherence observed by the other authors [18]. Further studies, such as cell culture tests, double hybrid

systems and electron microscopy, are needed to prove or disprove this hypothesis. The results showed some differences between the cytotoxic potential of *vly* from patients with severe chronic BV and from women with low expressed clinical symptoms, presumably due to a change in the amino-acid chain [18]. Vaginolysin alone is not responsible for all pathogenetic potentials in the BV etiology. The toxic potential changes, such as the biofilm formation and adhesion capacity, sialidase production and ability to degrade mucin, determine *G. vaginalis* as more virulent pathogen than other microbiota associated with BV [3, 8, 18].

In conclusion, three major groups were identified using phylogenetic analysis based on the whole *vly* gene and the fragment between the F1-GVaglysRLong primers. The observed distribution into the groups was mainly due to differences in amino-acid positions A32T N83D D112N, which is why the F1-GVaglysRLong primer pair can be considered appropriate for detection of the *vly* gene and for phylogenetic analysis. In addition, this primer pair could be employed together with the F1-GvaglysRshort primers for detection of the *vly* gene of *G. vaginalis* in isolates and clinical samples and the results could be used for systematic analysis of a possible link between the clinical manifestation and certain *G. vaginalis* phenotypes. This is the first study on the detection and phylogenetic characterization of the *vly* from *G. vaginalis* such as the leading factor of virulence related with the pathogenesis of BV in Bulgarian women.

Conflict of interest. All authors declare to have no conflict of interest.

REFERENCES

[1] Koneman E. W., S. D. Allen, W. M. Jand, P. C. Schreckenberger, C. W. Jr Washington (1997) Chapter 13, Microbiology, Irregular or coryneform nonspore forming bacilli. In Color Atlas and Textbook of Diagnostic 5th edition, 670–708, Edited by Koneman, EW, Allen, SD, Jand WM, Schreckenberger PC, Washington CW Jr. Lippincott, New York, USA, 651–708.

[2] Catlin B. W. (1992) Gardnerella vaginalis: characteristics, clinical considerations, and controversies. Clin. Microbiol. Rev., **5**(3), 213–237.

[3] Gilbert N. M., W. G. Lewis, A. L. Lewis (2013) Clinical features of bacterial vaginosis in a Murine model of vaginal infection with *Gardnerella vaginalis* PLOS One, **8**(3), e59539.

[4] Bretelle F., P. Rozenberg, A. Pascal, R. Favre, C. Bohec, A. Loundou, M. V. Senat, G. Aissi, N. Lesavre, J. Brunet, H. Heckenroth, D. Luton., D. Raoult, F. Fenollar Groupe de Recherche en Obstetrique Gynecologie (2015) High *Atopobium vaginae* and *Gardnerella vaginalis* vaginal loads are associated with preterm birth. Clin. Infect. Dis., **15**(60)(6), 860–867.

[5] Gergova R. T., T. V. Strateva, I. G. Mitov (2013) *Gardnerella vaginalis*-associated bacterial vaginosis in Bulgarian women, Braz. J. Infect. Dis., **17**(3), 313–318.

[6] Machado A., J. Castro, T. Cereija, C. Almeida, N. Cerca (2015) Diagnosis of bacterial vaginosis by a new multiplex peptide nucleic acid fluorescence in situ hybridization method, Peer J. **3**, e780. doi: 10.7717/peerj.780, 780 [pii], pmid:25737820.

[7] Awoniyi A. O., O. I. Komolafe, O. Bifarin, O. Olarinde (2015) Bacterial vaginosis among pregnant women attending a primary health care center in Ile-Ife, Nigeria, Adv. Res. J. Med. Med. Sci., **4**(1) 057–060.

[8] Udayalaxmi J., G. K. Bhat, S. Kotigadde (2011) Biotypes and virulence factors of *Gardnerella vaginalis* isolated from cases of bacterial vaginosis, Indian J. Med. Microbiol., **29**, 165–168.

[9] Gelber S. E., J. L. Aguilar, L. Kanako, T. Lewis, A. J. Ratner (2008) Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*, J. Bacteriology, **190**(11), 3896–3903.

[10] Nugent R. P., M. A. Krohn, S. L. Hillier (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation, J. Clin. Microbiol., **29**(2), 297–301.

[11] Edgar R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Research, **32**(5), 1792–1797.

[12] Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Molecular Biology and Evolution, **28**, 2731–2739.

[13] Posada D. (2008) jModelTest: Phylogenetic Model Averaging, Mol. Biol. Evol., **25**(7), 1253–1256.

[14] Abascal F., R. Zardoya, D. Posada (2005) ProtTest: Selection of best-fit models of protein evolution, Bioinformatics, **21**(9), 2104–2105.

[15] Sanchez R., F. Serra, J. Tárraga, I. Medina, J. Carbonell, L. Pulido, A. de María, S. Capella-Gutierrez, J. Huerta-Cepas et al. (2011) Phylemon 2.0: a suite of web-tools for molecular evolution, phylogenetics, phylogenomics and hypotheses testing, Nucleic Acids Res., **39**, (Web Server issue):W470-4.

[16] He Q., M. Marjamaki, H. Soini, J. Mertsola, M. K. Viljanen (1994) Primers are decisive for sensitivity of PCR, Biotechniques, **17**(1), 82–87.

[17] Sheffield V. C., D. R. Cox, L. S. Lerman, R. M. Myers (1989) Attachment of a 40 base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single base changes, Proc. Natl. Acad. Sci., **86**, 232–236.

[18] Harwich M. D .Jr., M. A. Joao, G. A. Buck, J. F. Strauss, J. L. Patterson, A. T. Oki, P. H. Girerd, K. K. Jefferson (2010) Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies, BMC Genomics, **11**, 375, doi: 10.1186/1471-2164-11-375.

Department of Medical Microbiology
Medical University of Sofia
Faculty of Medicine
1, St. G. Sofiiski Blvd
1431 Sofia, Bulgaria
e-mail: renigergova@mail.bg

View publication stats