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Diagnosis of bacterial vaginosis by a new multiplex peptide nucleic acid fluorescence *in situ* hybridization method

Bacterial vaginosis (BV) is one of most common vaginal infection and its diagnosis by classical methods reveals low specificity. Our goal was to compare the accuracy of BV diagnosis between the gold standard method, Nugent score, and our novel Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) methodology, which targets *Lactobacillus* and *Gardnerella vaginalis* populations. Epidemiological characteristic of the population under study (n=150) mirrored what has been described before in other major studies. Our results have shown a sensitivity of 84.6% (95% confidence interval (CI), from 64.3 to 95.0%) and a specificity of 97.6% (95% CI, from 92.6 to 99.4%), which attests the clinical value of this PNA-FISH approach. This methodology combines the specificity of PNA probes for *Lactobacillus* species and *G. vaginalis* visualization, and the criteria defined by Nugent score, allowing a trustful evaluation of the bacteria present in vaginal microflora and avoiding the occurrence of misleading diagnostics. Therefore, the PNA-FISH methodology represents a valuable alternative for BV diagnosis.

Diagnosis of Bacterial Vaginosis by a New Multiplex Peptide Nucleic Acid Fluorescence *In Situ* Hybridization Method

António Machado; Joana Castro; Tatiana Cereija; Carina Almeida; Nuno Cerca*

Centre of Biological Engineering, LIBRO – Laboratory of Research in Biofilms Rosário Oliveira, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.

* **Corresponding author:** nunocerca@ceb.uminho.pt

Tel.: (+351) 253 604 400

Fax: (+351) 253 604 429

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Introduction

Bacterial vaginosis (BV) often exhibits high prevalence, high relapse rates and associated complications, which renders this infection of global importance (Falagas, Betsi & Athanasiou, 2007; Tibaldi et al., 2009). BV is associated with increased taxonomic richness and diversity (Oakley et al., 2008) and is normally characterized by a decrease in vaginal lactobacilli and a simultaneous increase in the anaerobes population (Tibaldi et al., 2009). Therefore, vaginal bacterial communities differ dramatically between healthy patients and patients with BV, where *G. vaginalis* is present in over 90% of BV cases (Verstraelen & Swidsinski, 2013). *G. vaginalis* role is still controversial since this bacterium is also present in 10-40% of healthy women (Aroutcheva et al., 2001; Hickey & Forney, 2014; Silva et al., 2014); however, recent evidence suggests that the presence of *G. vaginalis* biofilms, instead of dispersed cells, are in fact an indication of BV (Verstraelen & Swidsinski, 2013). Furthermore, we recently demonstrated that *G. vaginalis* has significant higher virulence potential than other 29 BV associated species, including higher cytotoxicity and biofilm formation ability (Alves et al., 2014). We also provided evidence that *G. vaginalis* biofilms can establish synergistic relationships with other BV anaerobes (Machado, Jefferson & Cerca, 2013), further highlighting its pivotal role on BV development.

The most frequently used method for BV diagnosis is the physician's assessment by Amsel clinical criteria (Forsum, Hallén & Larsson, 2005). This method is fairly subjective and is based on the observation of the following symptoms: vaginal fluid with pH above 4.5; positive "whiff test" (detection of fishy odor upon 10% potassium hydrogen addition); presence of clue cells (vaginal epithelial cells covered by bacteria) on microscopic examination of vaginal fluid;

and homogeneous milky vaginal discharge. At least three of the four symptoms described above must be present to establish a positive BV diagnosis (Amsel et al., 1983). Despite the fact that the Amsel criteria does not require intensive training, it is not the most appropriate method to diagnose BV, due to its low specificity (Dickey, Nailor & Sobel, 2009).

Alternatively, laboratory diagnosis is based on the Nugent score analysis, a microscopic method that quantifies three different bacteria morphotypes presented in the vaginal smears (Nugent, Krohn & Hillier, 1991). These authors have created a Gram stain scoring system based on the evaluation of the following morphotypes: large gram-positive rods (*Lactobacillus* spp. morphotypes); small gram-variable rods (*G. vaginalis* morphotypes); small gram-negative rods (*Bacteroides* spp. morphotypes); and curved gram-variable rods (*Mobiluncus* spp. morphotypes). Each morphotype is quantified from 0 to 4 with regard to the number of morphotypes observed in the microscopic fields of the Gram-stained vaginal smear. The vaginal microflora is then classified in normal microflora (scores of 0 to 3) or as BV (scores of 7 to 10), based on the sum of each morphotype score (Livengood, 2009; Nugent, Krohn & Hillier, 1991). However, the evaluation of smears is also subjective and user dependent (Sha et al., 2005). Furthermore, due to its low specificity, the Nugent method also considers intermediate microflora whenever the final score is between 4 and 6.

Although both methodologies are easy and fast to perform, they do not provide a robust diagnosis of BV. When combined, these standard tests have a sensitivity and specificity of 81 and 70% (Forsum et al., 2005), respectively. To improve BV diagnosis, several new molecular methodologies have been proposed, being Fluorescence *in situ* hybridization (FISH) a very promising alternative. This technique combines the simplicity of microscopic observation and the specificity of DNA/rRNA hybridization, allowing the detection of selected bacterial species

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and morphologic visualization (Justé, Thomma, & Lievens, 2008; Nath, 2000). Nowadays, Peptide Nucleic Acid (PNA) probes are used instead of natural nucleic acids to improve FISH efficiency because they enable quicker and more specific hybridization (Lefmann et al., 2006; Peleg et al., 2009; Wilson et al., 2005). These types of probes, in which bases are linked by a neutral peptide backbone, avoid the repulsion between the negatively charged phosphate backbone characteristic of DNA/DNA hybridization (Stender et al., 2002). Since PNA is a synthetic molecule, probes are also resistant against cytoplasmic enzymes, such as nucleases and proteases (Amann & Fuchs, 2008). In addition, the hybridization step can be performed efficiently under low salt concentrations, which promotes the destabilization of rRNA secondary structures and consequently improves the access to target sequences (Almeida et al., 2009; Cerqueira et al., 2008). All these advantages have made PNA-FISH a new promising tool for diagnosis and therapy-directing techniques, providing already a rapid and accurate diagnosis of several microbial infections (Hartmann et al., 2005; Shepard et al., 2008; Søgaard et al., 2007; Trnovsky et al., 2008).

We have previously developed a multiplex PNA-FISH method, able to specifically quantify *in vitro* Lactobacilli spp. and *G. vaginalis* adhered to HeLa cells (Machado et al., 2013). To determine the feasibility of our novel PNA-FISH method as a diagnostic tool for BV, we have blind tested our multiplex methodology on vaginal samples from Portuguese women and compared those results with the laboratory microscopic derived method using the Nugent score.

Material & Methods

Vaginal sample collection and preparation

A total of 200 vaginal fluid samples were obtained, after informed consent, as approved by the Institutional Review Board (Subcomissão de Ética para as Ciências da Vida e Saúde) of University of Minho (process SECVS 003/2013). The vaginal samples were collected for Gram staining and FISH procedures, using the culture swab transport system (VWR, CE0344, Italy). These swabs were brushed against the lateral vaginal wall to collect the vaginal fluid sample, placed into the culture swab transport media and immediately stored at 4 ºC. First, the vaginal samples were used for Gram stain procedure, as described by Nugent and colleagues (Nugent et al., 1991). Next, swabs were immersed in 1 ml of phosphate buffer saline (PBS) and the remaining vaginal material collected by centrifugation at 17,000 *g* during 5 min at room temperature. Afterwards, the pellet was resuspended in 2 ml of saline solution (0.9%NaCl) and finally diluted 1:10 in saline solution or PBS to eliminate possible contaminants that could interfere with FISH procedures, as previously described (Machado et al., 2013).

Classification of vaginal smears according to Nugent score

Vaginal samples evaluation was performed using the Nugent criteria score (Nugent et al., 1991). Briefly, Gram stained vaginal smears were examined under oil immersion objective (1000x magnification) and 10-15 microscopic fields were evaluated for each sample. The composite score was grouped into three categories, scores 0-3 being normal, 4-6 being intermediate, and 7-10 being definite bacterial vaginosis. Finally, the smears that showed scores between 0-3 and 7-10 were selected for further study, as normal (–) and BV (+) samples, respectively. Meanwhile, the smears with a Nugent score of 4-6 or with incomplete epidemiological data were rejected from our study.

Fluorescent *in situ* hybridization

The 150 BV+ or BV- (as described above) vaginal samples were used on a blind PNA-FISH test. For each sample, 20 µL of the final suspension were spread on glass slides. The slides were air-dried prior to fixation. Next, the smears were immersed in 4% (wt/vol) paraformaldehyde (Fisher Scientific, United Kingdom) followed by 50% (vol/vol) ethanol (Fisher Scientific, United Kingdom) for 10 min at room temperature on each solution. After the fixation step, the samples were covered with 20 µL of hybridization solution containing 10% (wt/vol) dextran sulphate (Fisher Scientific, United Kingdom), 10 mM NaCl (Sigma, Germany), 30% (vol/vol) formamide (Fisher Scientific, United Kingdom), 0.1% (wt/vol) sodium pyrophosphate (Fisher Scientific, United Kingdom), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma, Germany), 0.2% (wt/vol) ficoll (Sigma, Germany), 5 mM disodium EDTA (Sigma, Germany), 0.1% (vol/vol) triton X-100 (Sigma, Germany), 50 mM Tris-HCl (at pH 7.5; Sigma, Germany) and 200 nM of each PNA probe (*Lactobacillus* spp. PNA Probe: Lac663 probe, Alexa Fluor 488-ACATGGAGTTCCACT; HPLC purified > 90%; *Gardnerella vaginalis* PNA Probe: Gard162 probe, Alexa Fluor 594-CAGCATTACCACCCG; HPLC purified > 90%). Subsequently, the smears were covered with coverslips and incubated in moist chambers at the hybridization temperature (60 ºC) during 90 min. Next, the coverslips were removed and a washing step was performed by immersing the slides in a pre-warmed washing solution for 30 min at the same temperature of the hybridization step. This solution consisted of 5 mM Tris base (Fisher Scientific, United Kingdom), 15 mM NaCl (Sigma, Germany) and 0.1% (vol/vol) triton X-100 (at pH 10; Sigma, Germany). Finally, the glass slides were allowed to air dry.

Fluorescence microscopic visualization and bacterial quantification

Prior to microscopy, one drop of non-fluorescent immersion oil (Merck, Germany) was added to either slides and covered with coverslips. Microscopic visualization was performed using an Olympus BX51 (Olympus Portugal SA, Portugal) epifluorescence microscope equipped with a CCD camera (DP72; Olympus, Japan) and filters capable of detecting the two PNA probes (BP 470-490, FT500, LP 516 sensitive to the Alexa Fluor 488 molecule attached to the Lac663 probe and BP 530-550, FT 570, LP 591 sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe).

In each experimental assay, a negative control was performed simultaneously, in which all the steps described above were carried out, but where no probe was added in the hybridization step. Finally, 20 random regions of each glass slide were photographed. All images were acquired using Olympus CellB software using a total magnification of ×1000.

Statistical analysis

The data was analyzed to calculate sensitivity, specificity, accuracy, positive and negative likelihood ratios (PLR and NLR, respectively) of the PNA-FISH methodology, with 95% confidence intervals (CI), using a clinical online statistical software (www.vassarstats.net/clin1.html; accessed 2014) (Senthilkumar, 2006). The classic Nugent criteria score was used as the diagnostic true.

Results and Discussion

On this prospective study, 150 vaginal samples were used to compare BV diagnosis by the classic Nugent criteria and our PNA-FISH methodology. As shown in Table 1, the main characteristics of the sample population used to validate our method, mirrors what has been described in other main epidemiological studies, namely (1) the overall rate of positive BV cases (17%) in the general population (Koumans et al., 2007; Li et al., 2014; Jespers et al., 2014), (2) an association between previous BV infections and BV positive diagnostic (Bilardi et al., 2013; Guedou et al., 2013), (3) a higher risk factors for women using the pill, instead of a condom (Bradshaw et al., 2013; Guedou et al., 2013), and (4) the history of previous pregnancy higher in women with BV (Africa, Nel & Stemmet, 2014; Mengistie et al., 2014).

As shown in Table 2, PNA-FISH method was able to diagnose 121 from a total of 124 healthy cases and capable to categorize 22 positive cases from a total of 26 BV cases, when compared with the standard Nugent score. PNA-FISH methodology was capable to illustrate clear differences between healthy and BV samples, showing specific detection of *Lactobacillus* spp. and *G. vaginalis* species directly in clinical samples. In fact, a typically healthy sample and a BV sample exhibited a totally different vaginal microflora, such as UM300 and UM235 samples, respectively, being clue cells and *G. vaginalis* augmentation easily detected in UM235 sample (see Fig. 1). However, some discrepancies were also found between the two methodologies, more exactly in 7 vaginal samples. In fact, 4 vaginal samples were positive for BV by Gram staining but negative by PNA-FISH evaluation while the others 3 vaginal samples were negative for BV by Gram staining but positive by PNA-FISH evaluation. It is well known that conventional BV diagnosis accuracy is highly dependent on the training and experience of the technician due to the unspecific staining of the Gram method (Simoes et al., 2006), which might explain some of the discrepant results observed.

To better evaluate the diagnostic value of the proposed PNA-FISH approach, the technique performance was assessed by determining the following parameters: specificity, sensitivity, accuracy, PLR, NLR. Based on these results, an experimental specificity of 97.6% (95% CI, 92.6 to 99.4%) and sensitivity of 84.6% (95% CI, 64.3 to 95.0%) were obtained for the BV diagnosis by our PNA-FISH method (Table 2). Therefore, a high accuracy was also obtained for our PNA-FISH method, more exactly, a value of 95.3% (95% CI, 89.2 to 98.3%).

Regarding the likelihood ratios the PNA-FISH method evidenced a PLR of 34.97 and a NLR of 0.16. So, the specificity and the NLR values show the test ability to correctly identify as normal person that do not have BV. While, the low NLR obtained, in fact, tells that the probability of having BV is much decreased (0.16) for a negative PNA-FISH result. Moreover, our experimental specificity revealed to be superior than Nugent's Gram stain system specificity (83%) (Schwebke et al., 1996). Therefore, our method was able to correctly identify 97.6% of those patients previously classified with normal vaginal flora making PNA-FISH a trustful method to ensure a healthy diagnosis and avoiding false positive results.

In opposition, the sensitivity and PLR values demonstrated a strong association between a positive result for BV diagnostic and the probability of the patient having indeed BV. In this case, the high PLR tells us how increased is the probability of having BV (35×), if the test result is positive. The sensibility value was in fact lower than expected, taking in consideration our previous *in vitro* experiments, where we have reached to a sensibility of 100% (95% CI, from 81.5 to 100.0%) (Machado et al., 2013). Despite the experimental sensitivity (84.6%) was slight lower than the specificity of the Gram staining by Nugent score (89%) (Schwebke et al., 1996), it was nevertheless higher than the Amsel criteria sensitivity (60%) determined by Gallo and colleagues (Gallo et al., 2011). It is important to refer that other bacterial species, with similar

Gram staining morphology, could be at high number in the samples leading to an incorrect classification of BV according to Nugent criteria. In fact, Verhelst and colleagues presented evidences that infers a lack of accuracy in the interpretation of the results in Gram stain by Nugent score in their clinical results (Verhelst et al., 2005). Forsum and colleagues also found discrepancies in scoring bacterial cell types, when pleomorphic lactobacilli and other kinds of bacteria could be regarded as *G. vaginalis* cells, leading to an incorrect BV diagnosis (Forsum et al., 2002; Schwiertz et al., 2006). Also, it is important to refer that *G. vaginalis* may vary in size and form, from round to more elongated, where there is no defined border to separate them from the lactobacilli morphotypes (Forsum et al., 2002), thus illustrating again problems in the accuracy of the smears interpretation. These facts suggest that the sensitivity value is likely to be underestimated.

Overall, despite the cost effective nature of the Nugent score, PNA-FISH appears to be an accurate method for detecting BV from vaginal samples, maintaining similar complexity as the previous standard method.

Conclusions

In conclusion, in this study we described the first PNA-FISH methodology applied for BV diagnosis, and the parameters evaluated have proved it potential as a diagnostic tool. The performance characteristics of this PNA-FISH method also suggest that it might be a reliable alternative to the Amsel criteria and Gram stain under Nugent score. Despite our sample size was somewhat small, the population at study was representative from what has been described by many other epidemiological studies, therefore validating this prospective study.

References

Africa CW, Nel J, Stemmet M. 2014. Anaerobes and bacterial vaginosis in pregnancy: virulence factors contributing to vaginal colonisation. *International Journal of Environmental Research and Public Health* **11:**6979-7000. doi: 10.3390/ijerph110706979.

Almeida C, Azevedo NF, Iversen C, Fanning S, Keevil CW, Vieira MJ. 2009. Development and application of a novel peptide nucleic acid probe for the specific detection of Cronobacter genomospecies (*Enterobacter sakazakii*) in powdered infant formula. *Applied and environmental microbiology* **75:** 2925–2930. doi:10.1128/AEM.02470-08.

Alves P, Castro J, Sousa C, Cereija TB, Cerca N. 2014. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *Journal of Infectious Diseases* **210:** 593-596.

Amann R, Fuchs BM. 2008. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nature Reviews Microbiology* **6:** 339–348. doi:10.1038/nrmicro1888

Amsel R, Totten PA, Spiegel CA, Chen KC, Eschen-bach DHK. 1983. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *American Journal of Medicine* **74:** 14–22.

Aroutcheva AA, Simoes JA, Behbakht K, Faro S. 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clinical Infectious Diseases* **33:** 1022-1027.

Bilardi JE, Walker S, Temple-Smith M, McNair R, Mooney-Somers J, Bellhouse C, Fairley CK, Chen MY, Bradshaw C. 2013. The burden of bacterial vaginosis: women's experience of the physical, emotional, sexual and social impact of living with recurrent bacterial vaginosis. *PloS one* **8**: e74378. doi: 10.1371/journal.pone.0074378.

Bradshaw CS, Vodstrcil LA, Hocking JS, Law M, Pirotta M, Garland SM, De Guingand D, Morton AN, Fairley CK. 2013. Recurrence of bacterial vaginosis is significantly associated with posttreatment sexual activities and hormonal contraceptive use. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America* **56**: 777-786. doi: 10.1093/cid/cis1030.

Cerqueira L, Azevedo NF, Almeida C, Jardim T, Keevil CW, Vieira MJ. 2008. DNA mimics for the rapid identification of microorganisms by fluorescence *in situ* hybridization (FISH). *International Journal of Molecular Sciences* **9:** 1944–1960. doi:10.3390/ijms9101944

Dickey LJ, Nailor MD, Sobel JD. 2009. Guidelines for the treatment of bacterial vaginosis : focus on tinidazole. *Therapeutics and Clinical Risk Management* **5:** 485–489.

Falagas ME, Betsi GI, Athanasiou S. 2007. Probiotics for the treatment of women with bacterial vaginosis. *Clinical Microbiology Infection* **13:** 657–664.

Forsum U, Hallén A, Larsson P. 2005. Bacterial vaginosis-a laboratory and clinical diagnostics enigma. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **113:** 153–161.

Forsum U, Jakobsson T, Larsson PG, Schmidt H, Beverly A, Bjørnerem A, Carlsson B, Csango P, Donders G, Hay P, Ison C, Keane F, McDonald H, Moi H, Platz-Christensen JJ, Schwebke J. 2002. An international study of the interobserver variation between interpretations of vaginal smear criteria of bacterial vaginosis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **110:** 811–818.

Gallo MF, Jamieson DJ, Cu-Uvin S, Rompalo A, Klein RS, Sobel JD. 2011. Accuracy of clinical diagnosis of bacterial vaginosis by human immunodeficiency virus infection status. *Sexually Transmitted Diseases* **38:** 270–274. doi:10.1097/OLQ.0b013e3181fce4eb

Guedou FA, Van Damme L, Deese J, Crucitti T, Becker M, Mirembe F, Solomon S, Alary M. 2013. Behavioural and medical predictors of bacterial vaginosis recurrence among female sex workers: longitudinal analysis from a randomized controlled trial. *BMC Infectious Diseases* **13**: 208. doi: 10.1186/1471-2334-13-208.

Hartmann H, Stender H, Schäfer A, Autenrieth IB, Kempf VA. 2005. Rapid Identification of Staphylococcus aureus in Blood Cultures by a Combination of Fluorescence *In Situ*

Hybridization Using Peptide Nucleic Acid Probes and Flow Cytometry. *Journal of Clinical Microbiology* **43:** 4855–4857. doi:10.1128/JCM.43.9.4855

Hickey RJ, Forney LJ. 2014. *Gardnerella vaginalis* does not always cause bacterial vaginosis. *Journal of Infectious Diseases* **210:** 1682-1683.

Jespers V, Crucitti T, Menten J, Verhelst R, Mwaura M, Mandaliya K, Ndayisaba GF, Delany-Moretlwe S, Verstraelen H, Hardy L, Buve A, van de Wijgert J. 2014. Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: a cross-sectional study. *PloS one* **9**: e109670. doi: 10.1371/journal.pone.0109670.

Justé A, Thomma BP, Lievens B. 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiology* **25:** 745–761. doi:10.1016/j.fm.2008.04.009

Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, Sutton M, Markowitz LE. 2007. The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sexually Transmitted Diseases* **34**: 864-869.

Lefmann M, Schweickert B, Buchholz P, Göbel UB, Ulrichs T, Seiler P, Theegarten D, Moter A. 2006. Evaluation of peptide nucleic acid-fluorescence *in situ* hybridization for

identification of clinically relevant mycobacteria in clinical specimens and tissue sections. *Journal of Clinical Microbiology* **44:** 3760–3767. doi:10.1128/JCM.01435-06

Li, XD, Wang CC, Zhang XJ, Gao GP, Tong F, Li X, Hou S, Sun L, Sun YH. 2014. Risk factors for bacterial vaginosis: results from a cross-sectional study having a sample of 53,652 women. *European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology* **33**: 1525-1532. doi: 10.1007/s10096-014-2103-1.

Livengood CH. 2009. Bacterial Vaginosis : An Overview for 2009. *Reviews in Obstetrics and Gynecology* **2:** 28–37.

Ma L, Lv Z, Su J, Wang J, Yan D, Wei J, Pei S. 2013. Consistent condom use increases the colonization of *Lactobacillus crispatus* in the vagina. *PLoS One* **8:**e70716. doi: 10.1371/journal.pone.0070716.

Machado A, Almeida C, Salgueiro D, Henriques A, Vaneechoutte M, Haesebrouck F, Vieira MJ, Rodrigues L, Azevedo NF, Cerca N. 2013. Fluorescence *In Situ* Hybridization Method Using Peptide Nucleic Acid Probes for rapid detection of *Lactobacillus* and *Gardnerella* spp. *BMC microbiology* **13:**82.

Machado A, Jefferson KK, Cerca N. 2013. Interactions between *Lactobacillus crispatus* and Bacterial Vaginosis (BV)-Associated Bacterial Species in Initial Attachment and Biofilm Formation. *International Journal of Molecular Sciences* **14:**12004-12.

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Menard JP, Fenollar F, Henry M, Bretelle F, Raoult D. 2008. Molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. *Clinical Infectious Diseases* **47:** 33–43. doi:10.1086/588661

Mengistie Z, Woldeamanuel Y, Asrat D, Adera A. 2014. Prevalence of bacterial vaginosis among pregnant women attending antenatal care in Tikur Anbessa University Hospital, Addis Ababa, Ethiopia. *BMC Research Notes* **7:**822. doi: 10.1186/1756-0500-7-822.

Nath JK. 2000. A review of fluorescence *in situ* hybridization (FISH): current status and future prospects. *Biotechnic & Histochemistry* **75:** 54–78.

Nugent R, Krohn M, Hillier S. 1991. Reliability of Diagnosing Bacterial Vaginosis Is Improved by a Standardized Method of Gram Stain Interpretation. *Journal of Clinical Microbiology* **29:** 297–301.

Oakley BB, Fiedler TL, Marrazzo JM, Fredricks DN. 2008. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Applied and Environmental Microbiology* **74:** 4898–909. doi:10.1128/AEM.02884-07

Peleg AY, Tilahun Y, Fiandaca MJ, D'Agata EMC, Venkataraman L, Moellering RC, Eliopoulos GM. 2009. Utility of peptide nucleic acid fluorescence *in situ* hybridization for rapid

detection of *Acinetobacter* spp. and *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology* **47:** 830–832. doi:10.1128/JCM.01724-08

Schwebke JR, Hillier SL, Sobel JD, McGregor JA. 1996. Validity of the vaginal gram stain for the diagnosis of bacterial vaginosis. *Obstetrics & Gynecology* **88:** 573–576.

Schwiertz A, Taras D, Rusch K, Rusch V. 2006. Throwing the dice for the diagnosis of vaginal complaints? *Annals of Clinical Microbiology and Antimicrobials* **5:** 1–7. doi:10.1186/1476-0711-5-4

Senthilkumar MP. 2006. Statistics on the web. *Annals of The Royal College of Surgeons of England* **88:** 325–332. doi:10.1308/003588406X106469

Sha BE, Chen HY, Wang QJ, Zariffard MR, Cohen MH, Spear GT. 2005. Utility of Amsel criteria, Nugent score, and quantitative PCR for *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Lactobacillus* spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *Journal of Clinical Microbiology* **43:**4607-12.

Shepard JR, Addison RM, Alexander BD, Della-Latta P, Gherna M, Haase G, Hall G, Johnson JK, Merz WG, Peltroche-Llacsahuanga H, Stender H, Venezia RA, Wilson D, Procop GW, Wu F, Fiandaca MJ. 2008. Multicenter evaluation of the *Candida albicans*/*Candida glabrata* peptide nucleic acid fluorescent *in situ* hybridization method for

simultaneous dual-color identification of *C. albicans* and *C. glabrata* directly from blood culture bottles. *Journal of Clinical Microbiology* **46:** 50–55. doi:10.1128/JCM.01385-07

Silva D, Henriques A, Cereija T, Martinez-de-Oliveira J, Miranda M, Cerca N. 2014. Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in Portuguese women and association with risk factors for bacterial vaginosis. *International Journal of Gynecology & Obstetrics* **124:**178-9.

Simoes JA, Discacciati MG, Brolazo EM, Portugal PM, Dini DV, Dantas MC. 2006. Clinical diagnosis of bacterial vaginosis. *International Journal of Gynecology & Obstetrics* **94:** 28–32. doi:10.1016/j.ijgo.2006.04.013

Søgaard M, Hansen DS, Fiandaca MJ, Stender H, Schønheyder HC. 2007. Peptide nucleic acid fluorescence *in situ* hybridization for rapid detection of *Klebsiella pneumoniae* from positive blood cultures. *Journal of Medical Microbiology* **56:** 914–917. doi:10.1099/jmm.0.46829-0

Stender H, Fiandaca M, Hyldig-Nielsen JJ, Coull J. 2002. PNA for rapid microbiology. *Journal of Microbiological Methods* **48:** 1–17. doi:10.1016/S0167-7012(01)00340-2

Tibaldi C, Cappello N, Latino MA, Masuelli G, Marini S, Benedetto C. 2009. Vaginal and endocervical microorganisms in symptomatic and asymptomatic non-pregnant females : risk factors and rates of occurrence. *Clinical Microbiology and Infection* **15:** 670–679.

Trnovsky J, Merz W, Della-Latta P, Wu F, Arendrup MC, Stender H. 2008. Rapid and accurate identification of *Candida albicans* isolates by use of PNA FISH Flow. *Journal of Clinical Microbiology* **46:** 1537–1540. doi:10.1128/JCM.00030-08

Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, De Backer E, Temmerman M, Vaneechoutte M. 2005. Comparison between Gram stain and culture for the characterization of vaginal microflora : Definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. *BMC Microbiology* **5:** 61. doi:10.1186/1471-2180-5-61

Verstraelen H, Swidsinski A. 2013. The biofilm in bacterial vaginosis: implications for epidemiology, diagnosis and treatment. *Current Opinion in Infectious Diseases* **26:** 86–89.

Wilson DA, Joyce MJ, Hall LS, Reller LB, Roberts GD, Hall GS, Alexander BD, Procop GW. 2005. Multicenter Evaluation of a *Candida albicans* Peptide Nucleic Acid Fluorescent *In Situ* Hybridization Probe for Characterization of Yeast Isolates from Blood Cultures. *Journal of Clinical Microbiology* **43:** 2909–2912. doi:10.1128/JCM.43.6.2909

Witt A, Petricevic L, Kaufmann U, Gregor H, Kiss H. 2002. DNA hybridization test: rapid diagnostic tool for excluding bacterial vaginosis in pregnant women with symptoms suggestive of infection. *Journal of Clinical Microbiology* **40:** 3057–3059.

423 Figures and Tables

424

425 **Table 1.** Characteristics of the population of study (n=150). The samples classification as normal
426 or BV was performed according the Nugent score.

Variables	Women with normal flora (n=124)	Women with BV (n=26)
Age (years)	30.2 ± 11.42	32.5 ± 9.7
Children (%)		
No	68.5	50.0
Yes	27.4	50.0
Pregnant women (%)	4.0	0.0
Previously diagnosed with bacterial vaginosis (%)	16.9	38.5
Contraception (%)		
No contraception	8.9	15.4
Pill	54.0	61.5
Condom	25.8	11.5
Other	12.1	15.4

427 Data are mean ± standard deviation or n (%).

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Table 2. Comparison between PNA-FISH method *versus* Gram staining using Nugent score criteria for BV diagnosis.

PNA-FISH results	Nugent results		
	BV+	BV -	Total
BV +	22	3	25
BV -	4	121	125
Total	26	124	150
Statistical analysis of PNA-FISH method			
	Estimated value	Lower limit	Upper limit
Sensitivity	**84.6%**	64.3%	95.0%
Specificity	**97.6%**	92.6%	99.4%
Accuracy	**95.3%**	89.2%	98.3%
Positive likelihood	**34.97**	11.30	108.24
Negative likelihood	**0.16**	0.06	0.39

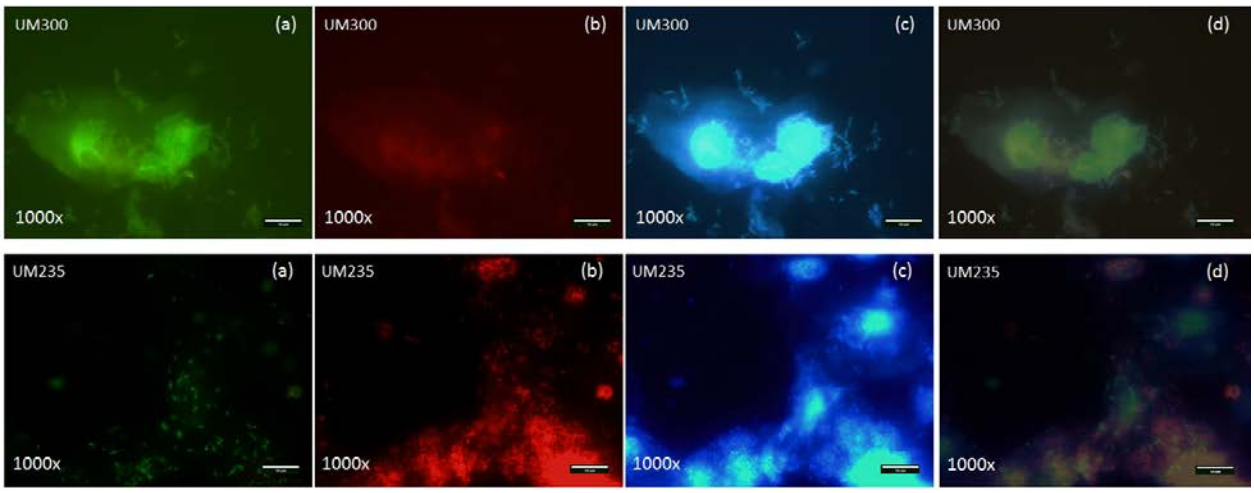

Figure 1 Fluorescence microscopy pictures of *Lactobacillus* spp., *Gardnerella vaginalis* and others bacteria species from a healthy (UM300) and BV (UM235) vaginal clinical samples by specific PNA probes (Lac663 and Gard162) associated with Alexa Fluor 488 and 594 fluorochromes and DAPI staining, respectively. (a) green filter; (b) red filter; (c) blue filter; (d) overlay of the three previous filters.