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An update on *Gardneralla vaginalis* associated bacterial vaginosis in Malaysia

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ABSTRACT

Objective: To update the status of *Gardnerella vaginalis* (*G. vaginalis*) as a causative agent of bacterial vaginosis (BV) in Malaysia and to define its epidemiology, metronidazole resistance and virulence properties.

Methods: It is a single-centre (Gynaecology clinic at the Hospital Kuala Lumpur, Malaysia) prospective study with laboratory-based microbiological follow up and analyses. Vaginal swabs collected from the patients suspected for BV were subjected to clinical BV diagnosis, isolation and identification of *G. vaginalis*, metronidazole susceptibility testing, vaginolysin and sialidase gene PCR, Piot's biotyping and amplified ribosomal DNA restriction analysis genotyping.

Results: Among the 207 patients suspected for BV, *G. vaginalis* was isolated from 47 subjects. *G. vaginalis* coexisted with *Trichomonas vaginalis* and *Candida albicans* in 26 samples. Three *G. vaginalis* isolates were resistant to metronidazole. Biotyping revealed 1 and 7 as the common types. Amplified ribosomal DNA restriction analysis genotype II was found to be more common (n = 22; 46%) than I (n = 12; 25.53%) and III (n = 13; 27.6%). All genotype I and III isolates carried the sialidase gene, while 91.6% and 84.6% contained the vaginolysin gene. Genotype I was significantly associated with post-gynaecological surgical complications and abortions (P = 0.002).

Conclusions: The existence of pathogenic *G. vaginalis* clones in Malaysia including drug resistant strains should not be taken lightly and needs to be monitored as these may bring more complications especially among women of child bearing age and pregnant women.

1. Introduction

Vaginosis is commonly defined as a pathological state characterized by the loss of normal vaginal flora, and overgrowth by other microbes including pathogenic parasites (*e.g. Trichomonas vaginalis* causing trichomoniasis), yeasts (*e.g. Candida albicans* causing candidiasis) and bacteria (*Gardnerella vaginalis*, *Bacteroides* spp., *Mobiluncus* spp., and *Mycoplasma hominis*) that results in vaginal discharge [1]. Among all pathogens, *G. vaginalis* supposedly plays a primary role in bacterial vaginosis (BV) and poses risk factors for poor obstetric and gynaecologic outcomes including preterm delivery, pelvic infection following gynaecologic surgery, development of vaginal infection after abortion and acquiring sexually transmitted diseases such as HIV [2,3]. The incidence of *G. vaginalis* is significantly higher among women with preterm labour and late miscarriage [4]. A recent systematic review reported BV prevalence vary between ethnic groups in North America, South America, Europe, the Middle East and Asia. The highest prevalence is seen in Africa and lowest in Asia and Europe [5].

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Bacterial vaginosis is treated with metronidazole or clindamycin orally or intra-vaginally. Resistance to metronidazole is not uncommon and yet is the standard frontline drug for treatment of BV [6]. Despite of treatment, in more than 50% of patients, symptoms return within one year [7]. In addition to drug resistance, failed restoration of lactobacilli may also play an important role in reoccurrence [8]. On the other hand, in a recent study, *G. vaginalis* of clades of 3 and 4 were found to be intrinsically resistant to metronidazole [9].

G. vaginalis being the key pathogen initiates BV by producing biofilms [2]. Biofilm is essential for their survival, enhancing crosstalk with other anaerobes and invading native species [10]. The challenge with multi speciated biofilm associated BV makes clinicians poorly armed with treatment choices [11].

Vaginolysin is an important pore forming toxin, that has the potential to lyse human red blood cell and vaginal epithelium resulting in tissue damage [12]. The neuraminidase sialidase produced by some strains of *G. vaginalis* interferes with host immune modulation resulting in adverse pregnancy outcomes [13].

In Malaysia, studies on BV are very limited in number. Literature search reported only one study on BV back in 1992 [14]. This study compared the occurrence of common microbes implicated in vaginal discharge among women with and without clinical complaints.

The main aim of the present study is to update the status of *G. vaginalis* associated BV in Malaysia. Different BV diagnostic methods, BV epidemiology, metronidazole/clindamycin resistance and virulence properties of *G. vaginalis* isolated from patients attending the gynaecology clinic in the largest tertiary care hospital in Malaysia are investigated here.

2. Material and methods

2.1. Ethics approval

The study protocol was performed according to the Helsinki declaration and was carried out after ethics approval from the Ministry of Health, Malaysia and from Universiti Putra Malaysia (NMRR-11-400-9394). Informed written consent was obtained from each subject upon agreement to participate in the study.

2.2. Clinical setting, study population and bacterial strains

This study was conducted in women patients who attended the gynaecology clinic of Hospital Kuala Lumpur (HKL) from 16th February to 20th July 2012. HKL is the largest government tertiary referral hospital in Kuala Lumpur, Malaysia, with 81 wards and 2 502 beds.

A total of 207 patients suspected for vaginosis were enrolled in the study; these patients either had abortion, vaginal discharge or postsurgical complications such as irritation, pain, discharge or infection. Patients who were menstruating, had other forms of vaginal bleeding or were pregnant were not included in the study.

2.3. Diagnosis for BV and isolation of G. vaginalis

BV was diagnosed based on the standard Amsel clinical criteria (wet mount test) and Nugent gram stain [15,16]. The clinical diagnosis for BV was defined as presence of at least three of the following criteria: homogeneous vaginal discharge, pH > 4.5, positive amine test, and presence of clue cells [15].

2.4. Amsel-criteria (wet mount test)

Samples of the vaginal discharge were collected using two dry cotton-wool tipped swabs. One swab was pressed briefly on a pH indicator paper (range 4.0–6.0) and then the same swab was suspended in two drops of normal saline solution in a glass tube. One drop of the mixture was placed on a glass slide and covered with a cover slip. The mixture was examined in a phase-contrast microscope at 400× magnification to identify clue cells (vaginal epithelial cells covered with gram negative coccobacilli) which suggested the presence of *G. vaginalis*. The same swab was then smeared on a glass slide and mixed with two drops of 10% potassium hydroxide solution for amine testing (Whiff test). A fishy odour on the Whiff test was considered an indicator for BV.

2.5. Nugent gram-stain

The second vaginal swab was subjected to direct gram-staining as described earlier [16]. The gram stain data were interpreted according to the following scheme: 1+, when one bacterium per field; 2+, 1 to 5 bacteria per field; 3+, 6 to 30 bacteria per field; 4+, more than 30 bacteria per field. Large gram-positive bacilli were assumed to be of the *Lactobacillus* morphotype. Smaller gram-variable bacilli were assumed to be of the *Gardnerella* morphotype. Other organisms were categorized by morphology, for *e.g.* gram-negative bacilli, curved rods, gram-positive cocci in chains, and fusiforms. When the *Lactobacillus* morphotype was present, the smear is interpreted as normal. When a mixed flora was found, containing *Gardnerella* morphotypes and other gram-negative and gram-positive bacteria at 1 to 2+ scores of the *Lactobacillus* morphotype, the smear was interpreted as providing a sign of BV. Prior to gram staining, the swab was streaked on horse blood agar plates (Oxoid, UK) and incubated at 37 °C in 5% CO_2 for 48 h and an extra 24 h for those cultures that did not show any growth during first 48 h of incubation. β-haemolytic transparent colonies indicated the presence of *G. vaginalis*, which was further identified based on gram stain morphology (showing small pleomorphic gram-variable rods), fermentation of starch and glucose but not mannitol, and lack of catalase and oxidase activity.

2.6. Identification of G. vaginalis by 16S rRNA gene PCR and sequence analysis

Isolates that were identified as *G. vaginalis* phenotypically were subjected to genotypic confirmation by *16S rRNA* gene sequencing after PCR [17].

2.7. Antibiotic susceptibility testing

All *G. vaginalis* isolates were tested for susceptibility to penicillin, ampicillin, vancomycin, clindamycin, gentamicin, nalidixic acid, rifampin, ciprofloxacin, cefuroxime, bacitracin, doxycycline and metronidazole by disk diffusion testing on Muller Hinton blood (MHB) agar. Susceptibility was evaluated by measuring the zone of inhibition after 48 h–72 h of growth. The data were interpreted according to the CLSI guidelines [18].

2.8. Biotyping of G. vaginalis by Piot's bio-typing scheme

All *G. vaginalis* isolates were typed by Piot's bio-typing scheme which is based on hippurate hydrolysis, β-galactosidase and lipase activity as described previously [19]. According to Piot's bio-typing scheme, *G. vaginalis* strains were classified into 8 biotypes based on the combination of biochemical reactions.

2.9. Genotyping of G. vaginalis by amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA of *G. vaginalis* clinical isolates was performed as described elsewhere [20], where the full length *16S rRNA* gene was amplified [21]. The PCR products were subjected to overnight restriction enzyme digestion using *Taq* I (AIT biotech Pte. Ltd., Singapore). The restriction patterns observed defined the genotype of *G. vaginalis* [14,21].

2.10. Virulence genes PCR

Genes coding for vaginolysin and sialidase were assessed as described earlier [12,13]. PCR products were identified by DNA sequencing (First Base Laboratories Sdn. Bhd., Malaysia).

2.11. Association of virulence genes, genotypes and biotypes with different gynaecological complications

In order to determine associations between *G. vaginalis* virulence and its bio/genotypes with gynaecological complications including postsurgical ones, vaginal discharge and abortion, statistical analysis was performed using SPSS, version 19. Kruskal Wallis Test was used to find statistically significant differences in the mean rank for each group. *P*-value less than 0.05 is considered as significant.

3. Results

3.1. Bacterial vaginosis and G. vaginalis isolation

Based on clinical observations (Amsels clinical criteria), among the 207 patients, 160 (77.2%) showed symptoms for bacterial vaginosis (positive for at least three criteria). The majority of these women produced characteristic vaginal discharge (n = 164; 78.7%), 145 (70%) had vaginal pH higher than 4.5, 134 (64.7%) showed a positive Wiff test and 64 (30.9%) exhibited clue cells. For Nugent gram staining, 64 (30.9%) women had score 4+ and 3+ consistent with bacterial vaginosis, while 143 (69.1%) showed score 0, 1+ and 2+ indicating normal bacterial vaginal flora. Among the 67 samples, who had clue cells and Nugent score of 4+ and 3+, 47 exhibited *G. vaginalis* morphotype colonies on blood agar and also showed positive biochemical reactions. Fourteen samples showed *Propionibacterium avidum* and three contained *Facklamia hominis* (data from hospital). All 47 isolates were confirmed as *G. vaginalis* by 16S rDNA sequence. The nucleotide sequence of the 16S rDNA gene was homologous to the GenBank sequence (Accession No: CP002104.1) and the 16S rRNA sequences of two isolates are deposited in GenBank under the accession numbers KC335149 and KC335150 respectively. Among the 47 *G. vaginalis* positive samples, 39 and 34 were co-infected with *Candida* spp. and *Trichomonas* spp., respectively. Twenty six samples contained all three pathogens.

3.2. Antibiotic susceptibility patterns

The antibiotic susceptibility test carried out for 47 isolates against 12 antibiotics showed different grades of resistance to different antibiotics. All isolates were susceptible to cefuroxime. Clindamycin and metronidazole which are given as the first line antibiotics for BV treatment in Malaysia showed susceptibility to 44 (93.6%) isolates. Susceptibility to other antibiotics were as follows: ampicillin (46; 97.9%), rifampin (46; 97.9%), vancomycin (37; 88.8%), penicillin (41; 87.2%), ciprofloxacin (40; 85.1%), bacitracin (36; 76.6%), nalidixic acid (23; 70.2%), gentamicin (22; 56.8%), and doxycycline (19; 40.4%).

3.3. G. vaginalis biotypes

Only 4 biotypes were found among the isolates studied here. Eighteen isolates belonged to biotype 1, 8 to biotype 5, 17 to biotype 7 and 4 were biotype 8. The most common biotypes were 1 and 7, while biotypes 2, 3, 4 and 6 were not detected.

3.4. G. vaginalis genotypes

PCR performed for forty-seven *G. vaginalis* isolates that were typed by ARDRA, produced a band at the expected size of 1 500 bp. Three genotypes I, II and III were observed after digestion with *Taq* I. Genotype II was more common (n = 22; 46%), while I and III were observed in 12 (25.53%) and 13 (27.6%) *G. vaginalis* isolates, respectively.

3.5. Biotype and genotype correlation

All biotype 7 isolates correlated with genotype II, the majority (n = 11; 61.1%) of biotype 1 strains were of genotype I. Biotype 5 shared genotype II (50%) and III (50%).

3.6. Virulence genes PCR

Twenty five and 26 *G. vaginalis* isolates carried the sialidase and *vly* genes, respectively. Twenty-one (44.6%) isolates harboured both sialidase and *vly* genes. All isolates that carried vaginolysin belonged to biotype 1 and 5, while isolates positive for sialidase belonged to biotype 1, 5 and 8. None of the biotype 2 carried either sialidase or vaginolysin genes.

3.7. Association of gynaecological complications with BV, Trichomoniasis and Candidiasis

As seen in Table 1, *G. vaginalis* isolated from patients who had abortion were either biotype 1 (Genotype I) or biotype 7 (Genotype II). Similarly, *G. vaginalis* (n = 10; 21%) isolated from patients who underwent gynaecological surgery such as hysterectomy, cervical lesion, cystectomy and patients with vaginal abscess are frequently associated with biotype 1, which carried sialidase and *vly* genes. Seventeen (36%) *G. vaginalis* isolated from patients who had vaginal discharge, belonged to genotype II, but were sialidase negative and 5 of them harboured

Table 1
Distribution of biotypes and genotypes among gynaecological complications.

Types	*n*	GS (*n* = 16)	VD (*n* = 29)	AB (*n* = 3)	GS/VD
Biotypes 1	18	10	6	2	1
Biotypes 5	8	1	6	0	–
Biotypes 7	17	5	13	1	1
Biotypes 8	4	0	4	0	–
Genotypes I	12	8	2	2	–
Genotypes II	22	5	17	1	1
Genotypes III	13	3	10	0	–

Table 2
Distribution of candidiasis and trichomoniasis among patients with gynaecological complications.

Infections	GS	Vaginal discharge	Abortion	GS/VD
Candidiasis	14/16	24/29	3/3	2/2
Trichomoniasis	13/16	19/29	2/3	0

the *vly* gene. Biotype 1/genotype I was significantly associated with post gynaecological surgical complications ($P < 0.05$). BV, trichomoniasis and candidiasis were found to be evenly distributed among all gynaecological complications (gynaecology surgery, vaginal discharge, abortion) (Table 2).

4. Discussion

In Malaysia, study on BV and its association with *G. vaginalis* is very limited. To our knowledge, this is the first study after 1992 on BV. Here, we have used a comprehensive approach covering the BV diagnosis, its prevalence, antibiotic susceptibility, biotypes and genotypes, virulence and its association with clinical complications. The main limitations would be relatively small sample size, lack of healthy controls, screening for other BV associated pathogens.

BV is a polymicrobial infection and *G. vaginalis* is considered as the indicator organism. The Amsel criteria established in 1983 [15] and the Nugent gram stain [16], are widely used for BV diagnosis. In the current study, based on Amsel clinical criteria, 160 (77.3%) cases of BV were identified, while Nugents gram stain showed positive indications for 64 (30.9%) cases. As reported in several studies, gram stain gives better assessment for BV due to superior sensitivity and reproducibility, while Amsel clinical criteria are cumbersome and insensitive as the genital culture of vaginal swab will contain several microbial species including the normal flora, not easily subjected to quality control and purely depend on the acumen of the clinician [22].

Although these non-culture-based methods are routinely used in many laboratories, the isolation of *G. vaginalis* is essential. Isolation and identification of *G. vaginalis* from a polymicrobial sample like vaginal swab containing vaginal microbiota is always challenging. With slow-growing and fastidious organisms, culture based identification is difficult and time consuming [23]. Hence in the present study, in addition to the routine phenotypic diagnosis, PCR based strategy using the 16S rRNA sequence was used as confirmatory diagnosis for *G. vaginalis*. As reported in a recent study [24], we also observed wet mount and gram staining as the most promising phenotypic methods for BV diagnosis. However, PCR based detection or quantitation is more rapid and adjunct to the complex phenotypic methods, which even identify patients at high risk for recurrent BV [25].

The co-infections of *G. vaginalis* with *Trichomonas* spp. and *Candida* spp. shows that vaginosis could be multi-pathogenic [26]. We found that candidiasis and trichomoniasis are also common among the Malaysian population and are evenly distributed with post gynaecological complications.

Piot's biotyping and ARDRA genotyping were used as additional tools to determine the epidemiology of *G. vaginalis*-associated BV. Although all genotypes and four biotypes were detected among Malaysian isolates, genotype I correlated with biotype 1 and genotype II correlated with biotype 7 were found to be more common among Malaysian strains. Similar studies in India also showed biotype 1 as the most prevalent (8; 25%), followed by biotype 2 (7; 21.9%) and biotypes 5 and 8 (5; 15.6%) [26].

Sialidase production which is an indicator of BV and strongly associated with adverse pregnancy outcomes including preterm labour is observed in all isolates of genotype I and III as previously reported [27]. The carriage of the sialidase gene in all isolates of genotype I and III confirms its clonal spread. The presence of the protein toxin vaginolysin, which belongs to the Cytolysin family that lyses erythrocytes and generates host immune responses, in the studied isolates reemphasize the pathogenic potential of *G. vaginalis*. Unlike sialidase, vaginolysin is not clonal and found to be carried in all genotypes. However, it is more common in genotypes I and III. As observed in the present study, the ability of *G. vaginalis* to elicit BV also depends on production of toxins.

Postoperative infections continue to be significant complications of major gynaecologic surgery. BV is one of the most well known complications of post gynaecologic surgery. The significant association of *G. vaginalis* isolates of biotype 1/genotype I that carry the genes coding for vaginolysin and sialidase with post gynaecological surgery infections and abortions once again emphasise the pathogenic potential of the virulence factors.

In conclusion, *G. vaginalis* is significantly associated with BV, co-infections with *Trichomonas* and *Candida* spp. are commonly seen. Genotype I and II strains which carry the virulence factors vaginolysin and sialidase are the predominant type in Malaysia and are significantly associated with post gynaecological surgical infections and abortions. Carriage of genes coding for sialidase is clonal. Resistance to first line antibiotics such as metronidazole and clindamycin should be closely monitored. The spread of highly pathogenic *G. vaginalis* clones in the hospital and the simultaneous emergence of drug resistant strains is of serious concern and needs to be strictly monitored as these may bring more complications especially among women of child bearing age and during pregnancy.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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