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SHORT COMMUNICATION

Characterization of *Gardnerella vaginalis* isolates: correlations among clades, biofilm formation and cytokine stimulation

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SUMMARY

We characterized 61 *Gardnerella vaginalis* (GV) strains isolated from women with bacterial vaginosis. GV clade 1 was the most commonly found (52.5%), followed by clade 4 (36.1%). All the strains were susceptible to ampicillin and clindamycin, whereas 96.7% and 6.6% of strains showed metronidazole and tetracycline resistance, respectively. Isolates within clade 4 tended to possess the highest ability to form biofilm. Strains resistant to metronidazole and tetracycline were all intermediate or high biofilm producers. All GV clades significantly upregulated the production of pro-inflammatory cytokines by HeLa cells, especially IL-8 and IL-6. Clade 4 induced a significantly higher production of IL-1β compared to other clades.

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Bacterial vaginosis (BV) is one of the most common disorders of the lower genital tract in women of reproductive age (Abou Chacra *et al*., 2022). This dysbiotic condition is characterized by the depletion of vaginal *Lactobacillus* species, with the subsequent increase of anaerobic and facultative bacteria such as *Gardnerella vaginalis*, *Fannyhessea vaginae*, and *Prevotella bivia* (Ceccarani *et al*., 2019). BV has been associated with several adverse health outcomes, including a higher risk of acquisition of sexually transmitted infections and pregnancy-related complications (Di Simone *et al*., 2020; Ma *et al*., 2012).

Although BV is considered a polymicrobial condition, *Gardnerella vaginalis* (GV) is often the predominant bacterial species, being frequently detected (up to 95% of cases) in vaginal samples of BV-affected women (Fredricks *et al*., 2005). GV exhibits a remarkable virulence potential compared to other BV-associated bacteria, including the production of the cholesterol-dependent toxin vaginolysin and sialidase enzymes (Patterson *et al*., 2010). Moreover, GV has a great ability to adhere to vaginal epithelial cells and form a biofilm, acting as a scaffold to which other anaerobic species (e.g., *Fannyhessea vaginae*) can subsequently attach (Swidsinski *et al*., 2014).

Genomic analyses based on the sequencing of a chaperonin-60 gene region have revealed that the GV population structure consists of four clades (Ahmed *et al*., 2012). Moreover, an emended description of *G. vaginalis* and descriptions of three new species – *Gardnerella leopoldii, Gardnerella piotii*, and *Gardnerella swidsinskii* – have recently been proposed (Castro et al., 2020).

It is still unclear and widely controversial whether the presence and the distribution of the different GV clades in the vaginal environment are universally applicable fingerprints of vaginal health or disease (Janulaitiene *et al*., 2017; Plummer *et al*., 2020). Moreover, there is no agreement on the correlations between the different GV clades and pathogenicity, in terms of production of virulence factors and ability to form biofilm.

Therefore, in this study we characterized a group of GV strains isolated from women with a dysbiotic vaginal condition. We assessed:

i) GV specific clade,
ii) the antimicrobial resistance pattern,
iii) the ability to form biofilm, and
iv) the capability to stimulate pro-inflammatory cytokines on epithelial cells.

A total of 61 GV strains were randomly selected from a collection of microorganisms available at the Bacteriology Unit of S. Orsola-Malpighi Hospital in Bologna (Italy).

Key words:
Gardnerella vaginalis, Bacterial vaginosis, Biofilm, Clades.

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Strains were isolated in the period January-September 2021 from vaginal samples submitted for routine diagnostic procedures. All vaginal specimens belonged to reproductive-age women with a condition of BV, identified by a microscopic Nugent score (NS) >7 (Zozaya-Hinchliffe *et al*., 2010).

Vaginal swabs (E-swab, Copan) were seeded on tryptic soy agar with 5% sheep blood and incubated for 48h at 37°C in 5% CO_2. After incubation, small gray translucent non-hemolytic colonies were isolated on chocolate agar and further incubated for 24 h at 37°C in 5% CO_2. GV strains were identified at the species level by means of MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany).

After nucleic acid extraction, each strain was tested against the four different GV clades by single-plex TaqMan real-time PCR assays, as previously described (Schuyler *et al*., 2016). Moreover, susceptibility to ampicillin, clindamycin, metronidazole, and tetracycline was evaluated for each strain by using E-test strips (BioMerieux, Marcy-l'Etoile, France) (Schuyler *et al*., 2016). Antimicrobial susceptibility testing was performed on chocolate agar and results were read after 48 h of incubation at 37°C in 5% CO_2. The following MIC breakpoints (μg/mL) were used to categorize resistance: metronidazole ≥32, ampicillin >2, clindamycin >4, tetracycline >64 (Petrina et al., 2017; CLSI, 2016).

To assess the ability of GV strains to form biofilm on an abiotic surface, a bacterial suspension with a final concentration of 10^7 colony forming unit (CFU)/mL in sterile saline was inoculated in polystyrene U-bottom 96-well plates (0.2 ml per well). Afterwards, bacteria were allowed to grow for 48h at 37°C in 5% CO_2. At the end of incubation, biofilm formation was evaluated by crystal violet staining as described by Abruzzo *et al.* (Abruzzo *et al*., 2018). At the end of the procedure, the OD values, measured at 595 nm, were used to categorize the isolates into four categories. GV strains were divided as follows: no biofilm producers (OD<0.1), weak biofilm producers (0.1≤ OD<0.5), intermediate biofilm producer (0.5≤ OD< 2), and high biofilm producers (OD>2).

Five representative strains of the most common GV clades (1, 2, 4) were randomly chosen to assess their ability to induce cytokine production on HeLa cells, used as a model of genital mucosa. HeLa cells (ATCC CCL-2) were grown to confluent monolayers in Dulbecco's minimal essential medium (DMEM; EuroClone, Pero, Italy), supplemented with 10% fetal bovine serum, and 1% L-glutamine 200 mM in 5% CO_2 at 37°C in 24-well plates.

For each strain, a bacterial suspension adjusted to 1×10^8 CFU/mL in DMEM supplemented with 10% fetal bovine serum was added to HeLa cells monolayers and incubated for 48h at 37°C. Afterwards, culture supernatants were collected for cytokine measurement. TNF-α, IL-6, IL-8, IL-1α and IL-1β concentrations were measured using Quantikine ELISA kits (R&D, Minneapolis, Minnesota, USA), following the manufacturer's instructions.

Statistical analyses were performed by GraphPad Prism software (version 5.02; GraphPad Software, San Diego, CA, USA). Fisher's exact test was used to compare categorical data, whereas one-way analysis of variance (ANOVA) test, followed by Tukey's multiple comparisons test was used for quantitative data. Statistical significance was determined at $P<0.05$.

The most common GV clade isolated was represented by clade 1 (32/61; 52.5%), followed by clade 4 (22/61; 36.1%), and 2 (6/61; 9.8%). Only one strain belonging to clade 3 was found (1/61; 1.6%).

All the strains were susceptible to ampicillin and clindamycin, whereas 96.7% (58/61) and 6.6% (4/61) of them showed metronidazole and tetracycline resistance, respectively (*Table 1*). Interestingly, all tetracycline-resistant strains harbored *tet(M)* gene (searched by an end-point PCR assay as described by Severgnini *et al*., 2021) and belonged to clade 4 (*Table 1*).

All GV strains were able to form biofilm, with significant variability among strains. Most GV strains (59%) were weak biofilm producers, whereas 24.6% were categorized as intermediate producers, and 16.4% were high biofilm producers. Isolates within clade 4 tended to possess the highest ability to form biofilm compared to the other clades (P=0.008; *Table* 1). Interestingly, GV strains resistant to metronidazole and tetracycline were all intermediate or high biofilm producers.

Overall, all GV clades caused a significant upregulation of all cytokines compared to untreated controls (*Figure 1*). The cytokine induced in highest quanti-

Table 1 - *Characteristics of the different GV clades.*

GV clade	*1* *(n=32)*	*2* *(n=6)*	*4* *(n=22)*	*P*
Metronidazole resistance	96.9% (31/32)	100% (6/6)	90.9% (20/22)	0.51
Tetracycline resistance	0% (0/32)	0% (0/6)	18.2% (4/22)	0.04 *
Biofilm formation (mean OD ± SD)	0.59 ± 0.48	0.55 ± 0.36	0.73 ± 0.54	0.008 **

Clade 3 was excluded from the analysis, being represented by only one strain. Fisher's exact test was used to assess statistical significance for categorical data (i.e., metronidazole and tetracycline resistance), whereas t-test for quantitative data (biofilm formation). *P<0.05, **P<0.01.

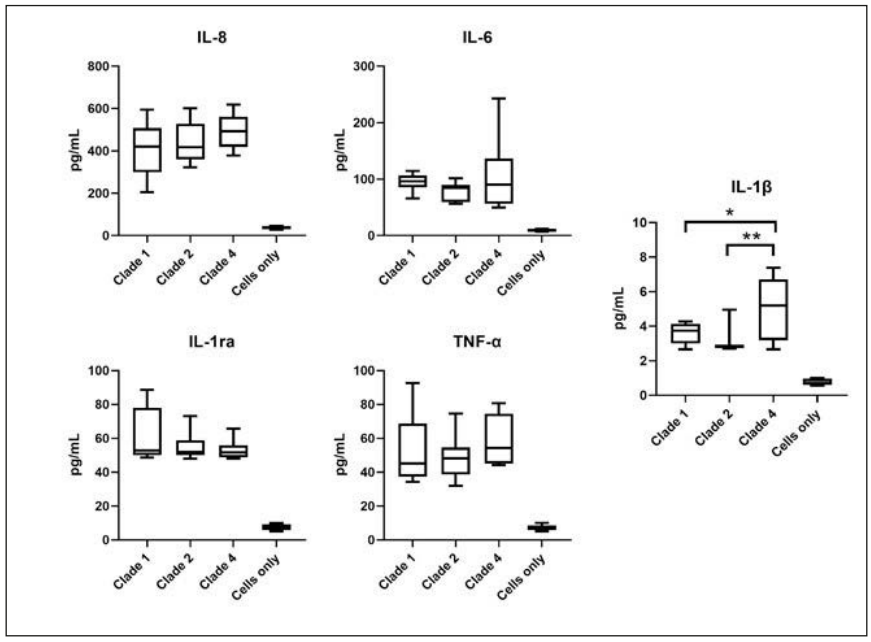

Figure 1 - *Cytokine production by HeLa cells in response to GV clades.*
*Data are presented as Tukey Box Plots. Boxes represent the interquartile ranges, lines within boxes represent medians and whiskers represent minimum and maximum values. Cytokine concentration is expressed as pg/mL. One-way analysis of variance (ANOVA) test, followed by Tukey's multiple comparisons was used to assess statistical significance; *P<0.05, **P<0.01. Clade 3 was excluded from the analysis, being represented by only one strain.*

ties was IL-8 (200-600 pg/mL), followed by IL-6 (50-250 pg/mL). IL-1β production was modulated in a different way by the various clades. Indeed, clade 4 induced a significantly higher production of IL-1β compared to both clade 1 (P=0.02) and clade 2 (P=0.001) (*Figure 1*).

To better understand the virulence/pathogenicity of the different GV clades, we studied 61 clinical strains isolated from women with a BV condition.

At first, we found that clade 1 represented more than 50% of strains recovered from the vaginal ecosystem of women with vaginal dysbiosis. This finding agrees with recent observations showing that clade 1 is significantly more common in samples with NS 7-10, in conjunction with a *Lactobacillus*-deficient vaginal microbiota (Janulaitiene *et al.*, 2017; Plummer *et al.*, 2020). Moreover, we detected only one strain belonging to clade 3, confirming its lower occurrence in the vaginal environment (Janulaitiene *et al.*, 2018).

We observed an extremely high rate of metronidazole resistance, exceeding 90%. It was previously shown that GV clades 3 and 4 are usually intrinsically metronidazole-resistant (Schuyler *et al.*, 2016). Nevertheless, in our collection, even strains belonging to clades 1 and 2 were often characterized by metronidazole MIC values>32 μg/mL. In line with this finding, even though metronidazole remains the most used antimicrobial agent for BV, more and more observations point out the limited in vitro activity of this antimicrobial against GV (Petrina *et al.*, 2017).

We can speculate that several factors could have contributed to the significant rate of metronidazole resistance, including high consumption of this drug for recurrent BV and frequent metronidazole self-medication for vaginal discharge (Löfmark *et al.*, 2010).

Four metronidazole-resistant strains showed a high level of resistance to tetracycline, attributed to the *tet*(M) conjugative transposon located on the chromosome (Severgnini *et al.*, 2021). All these strains belonged to clade 4 and were characterized by an intermediate-high ability to form biofilm. Considering that biofilm formation is associated with reduced ability of antimicrobials to eradicate GV, it will be interesting to evaluate the pathobiology of these strains by *in vitro* or *in vivo* models of BV with polymicrobial biofilms (Swidsinski *et al.*, 2011).

Even in the presence of significant strain variability, GV clade 4 was characterized by the highest ability to form biofilm. Our data are in contrast with the findings of Janulaitiene *et al.* demonstrating that the amount of GV biofilm did not differ significantly among clades and that the vast majority of clade 4 isolates formed little biofilm (Janulaitiene *et al.*, 2018). This discrepancy could be explained by several reasons, including a different selection of GV strains based on NS (e.g., in this study NS>7 vs NS>4 in the study by Janulaitiene).

In agreement with a recent study performed on vaginal epithelial cells, we found that GV causes significant upregulation of multiple inflammatory cytokines, especially for IL-8 (Manhanzva *et al.*, 2020).

We found, for the first time, that the ability of cytokine stimulation can be clade-specific, demonstrating the association between clade 4 and a higher production of IL-1β by HeLa cells. This aspect deserves particular attention if we consider that IL-1β induces a variety of proinflammatory events, being associated with microbial hydrolytic enzymes (e.g., sialidase) and a higher risk of obstetric complications (e.g., preterm birth) (Cauci *et al.*, 2008).

We are fully aware of some limitations of this study:

i) lack of detailed information on the women from whom the strains were isolated (e.g., symptoms, comorbidity, antibiotic therapy),
ii) potential loss of GV strains/clades by culture, considering that BV-women are more likely to have multiple clades of GV at the same time (i.e., co-colonization with more than two different GV clades) (Shipitsyna *et al*., 2019).

In conclusion, we added knowledge on the characteristics of GV clades in terms of virulence/pathogenicity, helping to elucidate BV pathogenesis. Further perspectives include the assessment of additional bacterial virulence factors, such as toxin vaginolysin and sialidase enzymes, as well as possible correlations between specific virulence factors and the clinical history of women from whom GV strains were isolated (e.g., vaginal symptoms, recurrence of BV, antibiotic use).

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