Identification of predominant culturable vaginal *Lactobacillus* species and associated bacteriophages from women with and without vaginal discharge syndrome in South Africa

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Lactobacillus jensenii, *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri* and *Lactobacillus vaginalis* were identified by 16S rRNA gene sequencing as the predominant culturable vaginal *Lactobacillus* species in a group of South African women, comprising 24, 22, 10, 10 and 9 %, respectively. A significant effect of vaginal discharge syndrome (VDS) and bacterial vaginosis (BV) on the distribution of predominant *Lactobacillus* species was observed. Whilst *L. crispatus* isolates were almost equally distributed between individuals with and without VDS and were not significantly reduced in women with BV versus normal microflora, *L. jensenii* isolates were significantly reduced in women with VDS ($P=0.022$) and reduced in women with BV versus normal microflora ($P=0.053$). Unlike *L. crispatus*, *L. jensenii* isolates were also significantly reduced in women with BV-associated VDS versus women without VDS and with normal microflora ($P=0.051$). In addition, lysogeny was commonly observed for *L. crispatus*, with 77 % of isolates yielding phage particles with contractile and non-contractile tails. Only 29 % of *L. jensenii* isolates yielded phage particles, and these were visible as tailless or podo-like particles.

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INTRODUCTION

Vaginal mucosal microfloras are typically dominated by Gram-positive *Lactobacillus* species, which serve as an important natural barrier against infection (Vásquez *et al.*, 2002; Iqbal & Kaul, 2008). The colonization of vaginal mucosa by engineered microbicide-secreting *Lactobacillus* strains is therefore seen as an economical and long-lasting means of enhancing this natural mucosal barrier for the prevention of human immunodeficiency virus (HIV) infection via vaginal intercourse (Liu *et al.*, 2006, 2008). However, to avoid potentially undesirable perturbations of the host's existing microflora by the introduction of exogenous *Lactobacillus* strains into the cervico-vaginal

mucosa, we have suggested that an alternative and potentially less disruptive strategy could be utilized. We have proposed that anti-HIV molecules could be expressed within the cervico-vaginal milieu by endogenous vaginal *Lactobacillus* populations that have been engineered *in situ* via plasmid transduction. To this end, we have recently successfully established that a predominant vaginal *Lactobacillus* species, *Lactobacillus gasseri*, can be transduced at high frequency, with Φ adh-derived transducing particles, resulting in transductants that stably express and secrete the HIV co-receptor antagonists CCL5 and CCL3 (Damelin *et al.*, 2010), thus supporting the use of transduction of endogenous vaginal *Lactobacillus* species as a potential strategy for the prevention of HIV infection across mucosal membranes. Encouraged by our findings, we have now sought to identify and develop novel

Abbreviations: BV, bacterial vaginosis; VDS, vaginal discharge syndrome.

transduction models for predominant vaginal *Lactobacillus* species within the South African population. Therefore, in this study, we isolated and characterized the predominant culturable vaginal *Lactobacillus* species and associated bacteriophages from pre-menopausal South African women and investigated the effect of vaginal discharge syndrome (VDS) and bacterial vaginosis (BV) on the distribution of predominant *Lactobacillus* species. To our knowledge, this is the first time that such a study has been undertaken in South Africa.

METHODS

Specimen collection. Vaginal swabs were collected from 144 pre-menopausal volunteers aged 18 and over, with a median age of 32 years, with ($n=75$) or without ($n=69$) VDS. VDS, as defined by the World Health Organization and South African syndromic management guidelines, is abnormal vaginal discharge, which may be accompanied by dysuria, vulval itching or vulval burning (WHO, 2003). Consecutive consenting women were recruited at two public service outpatient clinics in Johannesburg, South Africa, by clinical staff of the Sexually Transmitted Infections Reference Centre (NICD/NHLS). The study, including specimen collection, was approved by the University of the Witwatersrand's Human Research Ethics Committee (Medical) (protocol no. M080988). Vaginal swabs were used initially to produce a vaginal smear for subsequent Gram staining before being placed in anaerobic transport tubes (BBL Vacutainer Anaerobic Specimen Collectors; BD).

Bacterial culture. Upon arrival at the laboratory, on the same day or 18 h after collection swabs were streaked onto Rogosa, deMan–Rogosa–Sharpe (MRS) and Columbia agar plates with 5 % defibrinated sheep blood. Plates were incubated anaerobically for 48 h at 37 °C. Representative colonies were Gram-stained and those yielding Gram-positive/-variable rods were used to inoculate MRS broth or *Lactobacillus iners* broth (for colonies obtained from blood agar; ATCC Broth recipe no. 1685). Glycerol stocks (15 %) of culturable isolates were stored at –80 °C until further use.

Determination of BV. The Gram-stained vaginal smears obtained above were scored according to the method of Nugent *et al.* (1991) to detect normal microflora [Nugent score (NS) 0–3], intermediate flora (NS 4–6) and BV (NS 7–10).

16S rRNA gene sequencing. Isolates were identified to the species level using 16S rRNA gene sequencing as described previously (Yu *et al.*, 2009). Sequences were subjected to nucleotide–nucleotide BLAST and compared to known 16S rRNA genes in the public databases for species identification. The following 22 *Lactobacillus* ATCC reference strains were used to validate this method: *L. acidophilus* ATCC 4357; *L. brevis* ATCC 14869; *L. buchneri* ATCC 4005; *L. casei* subsp. *casei* ATCC 393; *L. crispatus* ATCC 33197; *L. crispatus* ATCC 33820; *L. delbrueckii* subsp. *lactis* ATCC 4797; *L. delbrueckii* subsp. *lactis* ATCC 12315; *L. fermentum* ATCC 11739; *L. fermentum* ATCC 14931; *L. gasseri* ATCC 9857; *L. iners* ATCC 55195; *L. jensenii* ATCC 25258; *L. johnsonii* ATCC 33200; *L. minutus* ATCC 33267; *L. paracasei* subsp. *paracasei* ATCC 27216; *L. plantarum* ATCC 14917; *L. reuteri* ATCC 23272; *L. rhamnosus* ATCC 21052; *L. ruminis* ATCC 25644; *L. salivarius* subsp. *salicinius* ATCC 11742; and *L. vaginalis* ATCC 49540.

Isolation of bacteriophages and electron microscopy. Overnight MRS broth cultures of *L. crispatus* and *L. jensenii* isolates were diluted to an OD₅₉₀ of 0.1, induced with 0.4 µg mitomycin C ml^{–1} for 18 h and centrifuged at 3000 g for 30 min. Putative induced phages in the

resultant supernatants were precipitated with polyethylene glycol and pelleted by centrifugation, as described previously (Damelin *et al.*, 2010). Resuspended putative phage pellets were placed on Formvar-coated copper grids, negatively stained with 2 % phosphotungstic acid, viewed with a transmission electron microscope at 80 kV [FEI Tecnai Biotwin Spirit TEM (120 kV)] and photographed with a MegaView III camera.

Statistical analysis. Associations were analysed using Fisher's exact test, with the level of statistical significance set at $P \leq 0.05$.

RESULTS

From 144 swabs, 115 isolates were cultured, with 36 % of individuals (52/144) yielding culturable lactobacilli and six individuals yielding two culturable *Lactobacillus* species (Table 1). *Lactobacillus* species made up 50 % of the culturable isolates, of which *L. jensenii* and *L. crispatus* were the predominant species (24 and 22 %, respectively; Table 1). *L. iners* and *L. gasseri* were also prevalent, each making up 10 % of the total *Lactobacillus* isolates obtained, whilst *L. vaginalis* contributed 9 % of the total lactobacilli (Table 1). Of the 57 non-*Lactobacillus* culturable bacteria, *Gardnerella vaginalis* was predominant (22 % of total isolates and 44 % of non-*Lactobacillus* isolates), whilst

Table 1. Predominant vaginal bacterial species from South African adult females

Species	No. of individuals with the indicated species*
<i>Lactobacillus acidophilus</i>	1
<i>Lactobacillus casei</i>	1
<i>Lactobacillus coleohominus</i>	1
<i>Lactobacillus crispatus</i>	13
<i>Lactobacillus gasseri</i>	6
<i>Lactobacillus iners</i>	6
<i>Lactobacillus ingluviei</i>	1
<i>Lactobacillus jensenii</i>	14
<i>Lactobacillus mucosae</i>	3
<i>Lactobacillus murinus</i>	1
<i>Lactobacillus oris</i>	1
<i>Lactobacillus reuteri</i>	2
<i>Lactobacillus salivarius</i>	3
<i>Lactobacillus vaginalis</i>	5
<i>Streptococcus anginosus</i>	14
<i>Gardnerella vaginalis</i>	25
<i>Actinomyces urogenitalis</i>	2
<i>Staphylococcus epidermidis</i>	1
<i>Atopobium vaginae</i>	1
<i>Aerococcus christensenii</i>	1
Unidentifiable vaginal species	11
Unidentifiable <i>Gardnerella</i> species	2

*Six individuals harboured two *Lactobacillus* species: one individual had *L. crispatus* and *L. iners*, one had *L. crispatus* and *L. salivarius*, three had *L. crispatus* and *L. jensenii*, and one had *L. jensenii* and *L. salivarius*.

Table 2. Effect of Nugent score (NS) and VDS on *Lactobacillus* isolate frequency, and distribution of phage-yielding isolates

Numbers in parentheses represent the number of phage-yielding isolates for each category.

Species	No. of individuals yielding the indicated species with NS of:			No. of individuals yielding the indicated species with or without VDS	
	0–3	4–6	7–10	VDS	No VDS
<i>L. acidophilus</i>	1				1
<i>L. casei</i>	1				1
<i>L. coleohominus</i>		1		1	
<i>L. crispatus</i>	9 (6)	1 (1)	3 (3)	6 (5)	7 (5)
<i>L. gasseri</i>	2	4		2	4
<i>L. iners</i>	6			3	3
<i>L. ingluviei</i>	1				1
<i>L. jensenii</i>	11 (3)	2 (1)	1	3	11 (4)
<i>L. mucosae</i>	2		1	2	1
<i>L. murinus</i>		1		1	
<i>L. oris</i>	1			1	
<i>L. reuteri</i>	1	1		2	
<i>L. salivarius</i>	3			1	2
<i>L. vaginalis</i>	2	1	2	2	3

Streptococcus anginosus accounted for 12 % of total isolates and 25 % of non-*Lactobacillus* isolates (Table 1).

Microscopy of the vaginal smears indicated that 48 % of women had normal microflora, 26 % had intermediate flora and 26 % had BV. Unsurprisingly, 69 % of culturable *Lactobacillus* species were obtained from individuals with normal microflora, whilst 19 and 12 % of *Lactobacillus* species were obtained from individuals with intermediate flora and BV, respectively (Table 2), with *L. crispatus* accounting for 43 % of lactobacilli obtained from individuals with BV (Table 2). We did not observe any

significant effect of HIV status on the abundance or distribution of *Lactobacillus* species within the cohort; however, we did observe a significant effect of VDS and BV on the distribution of predominant *Lactobacillus* species. Whilst *L. crispatus* isolates were almost equally distributed between individuals with and without VDS and were not significantly reduced in women with BV versus normal microflora, *L. jensenii* isolates were significantly reduced in women with VDS ($P=0.022$) and significantly reduced in women with BV versus those with normal microflora ($P=0.053$) (Table 2). Furthermore, unlike *L. crispatus*, *L. jensenii* isolates were significantly reduced in women

Fig. 1. Electron micrographs of bacteriophages from *L. crispatus* and *L. jensenii* isolates. (a, b) Putative *L. crispatus* siphoviruses with long, non-contractile tails. (c) Putative *L. crispatus* myovirus with contractile tail. (d) Multiple capsid or podo-like particles observed for a representative *L. jensenii* isolate. Bars, 25 nm.

with BV-associated VDS versus women without VDS and with normal microflora ($P=0.051$).

Next, we sought to identify endogenous phages in *L. jensenii* and *L. crispatus*, the predominant culturable vaginal *Lactobacillus* species identified. Lysogeny was commonly observed for *L. crispatus* with 10 of the 13 isolates (77%) yielding phage particles with contractile and non-contractile tails (Fig. 1a–c). Only four of the 14 *L. jensenii* isolates (29%) yielded phage particles; these were visible as tailless or podo-like particles (Fig. 1d).

DISCUSSION

Our findings indicated that the culturable vaginal *Lactobacillus* species predominant among women in South Africa (*L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*) are similar to those found to predominate in American and European studies (Antonio *et al.*, 1999; Kiliç *et al.*, 2001; Vásquez *et al.*, 2002; De Backer *et al.*, 2007).

We also observed an apparent increase in persistence for *L. crispatus* isolates over *L. jensenii* isolates in individuals with BV or VDS, as the former were isolated in equal frequency from individuals with VDS and were the most frequent isolates in individuals with BV, whilst *L. jensenii* isolates were significantly less recoverable from individuals with VDS and BV than those without. As a high degree of lysogeny (77%) was observed for vaginal *L. crispatus* strains, and as it has been established that prophages contribute directly to host survival in unfavourable environments by conferring survival phenotypes to lysogens (Chibani-Chennoufi *et al.*, 2004; Paul, 2008; Schuch & Fischetti, 2009), it is tempting to speculate that some of the mechanisms that allow the persistence of *L. crispatus* strains within the perturbed vaginal milieu of individuals with VDS and BV may be phage-conferred. In support of this speculation is the finding that *Streptococcus pneumoniae* lysogens harbouring the MM1 phage exhibit improved adherence to epithelial cells, potentially allowing increased persistence within the nasopharynx (Loeffler & Fischetti, 2006). However, further research would be required to determine whether analogous phage-conferred phenotypes exist for vaginal *L. crispatus* strains. In any event, our findings suggest that, due to its high frequency of lysogeny and persistence within individuals with VDS and BV, *L. crispatus* represents the most favourable vaginal *Lactobacillus* isolate for the development of novel transduction models specific for the South African population.

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