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Original article

Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial

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Abstract

This study enrolled 125 premenopausal women diagnosed with bacterial vaginosis (BV) by presence of vaginal irritation, discharge and 'fishy' odor, and Nugent criteria and detection of sialidase enzyme. The subjects were treated with oral metronidazole (500 mg) twice daily from days 1 to 7, and randomized to receive oral *Lactobacillus rhamnosus* GR-1 (1×10^9) and *Lactobacillus reuteri* RC-14 (1×10^9) or placebo twice daily from days 1 to 30. Primary outcome was cure of BV as determined by normal Nugent score, negative sialidase test and no symptoms or signs of BV at day 30. A total of 106 subjects returned for 30-day follow-up, of which 88% were cured in the antibiotic/probiotic group compared to 40% in the antibiotic/placebo group ($p < 0.001$). Of the remaining subjects, 30% subjects in the placebo group and none in the probiotic group had BV, while 30% in the placebo and 12% in the probiotic group fell into the intermediate category based upon Nugent score, sialidase result and clinical findings. High counts of *Lactobacillus* sp. ($>10^5$ CFU/ml) were recovered from the vagina of 96% probiotic-treated subjects compared to 53% controls at day 30. In summary, this study showed efficacious use of lactobacilli and antibiotic in the eradication of BV in black African women.

Keywords: Antibiotics; Probiotics; Vaginosis

1. Introduction

Bacterial vaginosis (BV) is found in women worldwide, with a particularly high prevalence in black women. Traditionally, symptomatic BV has been diagnosed clinically by presence of a vaginal discharge with a fishy odor, pH greater than 4.5, presence of densely colonized vaginal 'clue cells' and a heavy growth of Gram-negative anaerobes [1].

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However, odor, discharge and clue cells are not always found in patients diagnosed with BV [2], and this traditional diagnostic method is believed, by some, to be unreliable. The recognition that BV is associated with loss or depletion of indigenous lactobacilli led to the development of a Gram stain method of diagnosis [3]. Many groups have utilized this method effectively. A commercially available diagnostic system, the BVBlue test [4], has been introduced and found to be effective. It is based on the fact that sialidase is produced by BV organisms [5]. In the present study, it was decided to only recruit patients who had BV as diagnosed by the Nugent and BVBlue diagnostic methods as well as the presence of clinical symptoms and signs of BV. This was done to avoid any question of whether or not enrolled subjects had BV.

Lactobacillus rhamnosus GR-1 and *Lactobacillus reuteri* RC-14 were selected because of their documented ability to colonize the vagina [6] and their availability in a consistent, reliable capsule formulation that allows storage without the need for refrigeration [7]. The RC-14 strain was originally identified in 1986 as *Lactobacillus acidophilus* using biochemical tests. In 1998, it was reclassified as *Lactobacillus fermentum* RC-14 using ribotyping, before recently being identified by DNA–DNA hybridization as *L. reuteri* RC-14.

The decision to undertake the study in Nigeria was based upon three considerations: (1) A number of studies have emerged from this centre indicating an interest in, and potential application of, probiotic therapies in a region where no current commercial products are available, and in which the local investigators have no commercial interest in any particular product's outcome. (2) HIV is spreading rapidly in this region, and the fact that BV significantly increases the risk of viral acquisition [8], suggested a potential for improving the treatment results for BV and perhaps having an impact on the HIV pandemic. (3) Higher rates of BV have been found in some black populations [9], and it was felt that studying women in Nigeria would provide insight into whether or not the combination of antibiotic and probiotics has any potential clinical applicability.

2. Materials and methods

2.1. Probiotic strains

L. rhamnosus GR-1 and *L. reuteri* RC-14 were provided by Chr Hansen, Horsholm, Denmark, in gelatin capsules manufactured under FDA good manufacturing practices. Each capsule contained 1×10^9 viable cells of each strain. These probiotic organisms have previously been shown to colonize the vagina following oral intake [10], displace BV pathogens [11] and kill HIV [12].

2.2. Subjects and recruitment

A sample size of 60 subjects per group was calculated on the premise that there would be a 40% difference between the active and placebo groups. A total of 125 premenopausal women, aged 18 to 44 years, fitted the entry criteria of having symptoms and signs of BV, and a positive Nugent and BVBlue test score. They were enrolled from Benin City metropolis between January and June 2005. The patients presented with vaginal irritation and clinical symptoms and signs of BV, namely discharge with a 'fishy' odor. It was not possible to perform pH assessment at the sites, but two vaginal swabs were collected by a clinician or nurse using a sterile speculum, and Gram stained and found to have a Nugent score of 7–10 (few or no lactobacilli and dense Gram-negative rods adherent to cells) [3], as well as elevated sialidase activity as detected by a positive BVBlue test result [4]. Exclusions included pregnancy, lactation, evidence of gross inflammatory genital processes including any sexually transmitted infection (STI), use of systemic or intravaginal antibacterial agents currently or within the previous 14 days, use of investigational drugs within 30 days, hypersensitivity to metronidazole, warfarin, lithium or disulfiram and menstruation at time of diagnosis. The subjects were known to the referring physicians, and of the ones recruited, none had any evidence of STIs. All were known to be HIV negative, and no additional testing for the virus was performed at the time of trial recruitment. Each subject consented to the study after thorough explanation by the clinician, nurse or laboratory scientist. The Human Ethics Review Board at the University of Benin gave approval for the study.

2.3. Nugent scoring

Vaginal swab smears were graded by a technician blinded to the subject groups, on a 10-point scale based on the presence or absence of *Lactobacillus* morphotypes under oil immersion (×1000 magnification) as described previously [3]. A score of 0–3 was interpreted as consistent with a normal Gram-positive rod-dominated microbiota, a score of 4–6 as intermediate, and a score of 7–10 was considered consistent with BV-like conditions in which the samples were dominated by small Gram-negative and Gram-variable straight and curved rods.

2.4. BVBlue test

A second vaginal swab was placed in the BVBlue vial (Gryphus Diagnostics, L.L.C., Birmingham, AL) containing the chromogenic substrate of bacterial sialidase, and a laboratory timer was started. Two drops of BVBlue developer solution were added at 10 min: a blue–green color was recorded as a positive result and a yellow color was recorded as a negative result. The BVBlue test was performed at room temperature (25–28 °C).

2.5. Randomization

Over 500 subjects were screened, from which 125 were identified as suitable for the study. Randomization was performed using a computer-generated scheme, prepared by the pharmacy. The subjects were allotted numbers, and identical-looking

probiotic and placebo capsules were prepared and distributed in numbered containers, again by the pharmacy. The patients who satisfied the Nugent criteria for BV (7–10) and positive BVBlue test were identified to a central clinic, where they were randomized in a double-blind manner and given one oral dose of metronidazole (500 mg) twice daily for 7 days, plus either oral *Lactobacillus* GR-1 and RC-14 or placebo capsules (cellulose, magnesium stearate) twice daily for 30 days starting on day 1 of metronidazole treatment. The probiotics were taken at least 1 h after the antibiotic, although the two strains appear resistant. The patients were instructed to return the empty antibiotic container and probiotic alotubes at the end of treatment, at which time three vaginal swabs were collected, processed as previously described and evaluated for BV status. None of the subjects claimed to practice douching, but they were instructed not to do so during the study.

2.6. Primary outcome

The primary outcome of the study was cure of BV as determined by normal Nugent score, absence of clue cells, negative sialidase test and no symptoms or signs (no discharge or odor) of BV at day 30.

2.7. Recovery of Lactobacillus from the vagina

The third swab collected on day 30 was vigorously agitated in sterile 1 ml of phosphate buffered saline (PBS) (pH 7.1) to dislodge cells. Aliquots of each sample (0.1 ml) were serially diluted in PBS, and dilutions were plated in duplicate on MRS (de Man Rogosa Sharpe- Lab M, IDG, UK) agar plates with or without 50 μg/ml of tetracycline (Pfizer Nig. PLC) to aid in the selection of *L. rhamnosus* GR-1 and *L. reuteri* RC-14. The plates were incubated anaerobically using the BBL GasPack system at 37 °C for 48 h. The mean number of pale straw-colored colonies was counted and identified as presumptive *Lactobacillus* GR-1/RC-14; each colony type was Gram stained and tested for a catalase-negative reaction. Based upon previous studies of vaginal lactobacilli, the use of tetracycline was found to be helpful, albeit not 100% accurate, in differentiating the probiotic strains from indigenous strains.

3. Results

Of the 125 premenopausal women who enrolled and started the BV treatment, 84.8% (106) returned for the 30-day follow-up visit, and all provided evidence that they complied and took the antimicrobial drug and probiotics as required. Only three subjects in the placebo group did not return on day 30, whereas 16/65 who received probiotics did not return. The traditional reason for patients not returning for follow-up is that they feel well and have no recurrence of their initial symptoms. This finding was confirmed in several patients who did not return but who were traced by the clinical staff. This is suggestive of BV cure, particularly in the probiotic group, but nevertheless, none of these subjects were included in the final analysis.

No adverse effects were reported, although two subjects in the antibiotic/probiotic group reported persistent headaches that resolved after 3 days of therapy and they also indicated an increased appetite for the first 5 days of treatment.

All the subjects in both groups had clue cells and Nugent scores indicative of BV, and all had positive BVBlue tests at the beginning of the study, while at the 30-day follow-up, 43 of the 49 patients (88%) in the antibiotic/probiotic-treated group had normal Nugent scores and negative sialidase results, compared to 23 of 57 patients (40%) in the antibiotic/placebo group ($p < 0.001$) (Table 1; Fig. 1). Of the remaining antibiotic/probiotic subjects (12%), none had BV, but all had mild irritative symptoms, no discharge or odor, a weakly positive sialidase score and intermediate Nugent score. This contrasted with the remaining 34 antibiotic/placebo subjects, of which half had BV and the other half had an intermediate status. In short, 100% of the probiotic-treated patients no longer were diagnosed with BV, while 30% of the placebo group were positive.

Although cultures cannot detect all anaerobic lactobacilli such as *Lactobacillus iners* [11], the findings here did show a significant recovery of lactobacilli from zero to 96% (counts $> 10^5$ CFU/ml) in the antibiotic/probiotic-treated group (comparing days 0 and 30) compared to only 53% of the control group. This coincided with patients who were cured of BV or who had an intermediate status. No specific DNA probes are available for *Lactobacillus* GR-1 or RC-14. The antibiotic-treated agar can help to recover these organisms, but they were clearly not present in the placebo-treated subjects, and so we cannot conclude that all the lactobacilli found in the probiotic-treated group were strains GR-1 and RC-14. Nevertheless, eradication of BV did coincide with some recovery of the indigenous lactobacilli, confirming the Gram stain Nugent findings.

Table 1
Results of clinical study

Criteria	Metronidazole (1 g, days 1–7) plus *Lactobacillus* (days 1–30)		Metronidazole (1 g, days 1–7) plus placebo (days 1–30)	
	Day 0 65	Day 30 49	Day 0 60	Day 30 57
Positive clinically (discharge and 'fishy' odor)	65	5[a] $p = 0.005$ compared to placebo	60	19
Positive BVBlue test (sialidase)	65	6[b] $p = 0.035$ compared to placebo	60	17
Positive Nugent score (7–10 representative of BV)	65	0 $P = 0.003$ compared to placebo	60	17

Figures = number of subjects. Patients were diagnosed based on clinical symptoms and two diagnostic systems, then treated with metronidazole plus 2×10^9 *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or placebo.

[a] Mild irritative symptoms but no discharge or odor.

[b] Weakly positive. Statistical analysis using Fisher's exact tests.

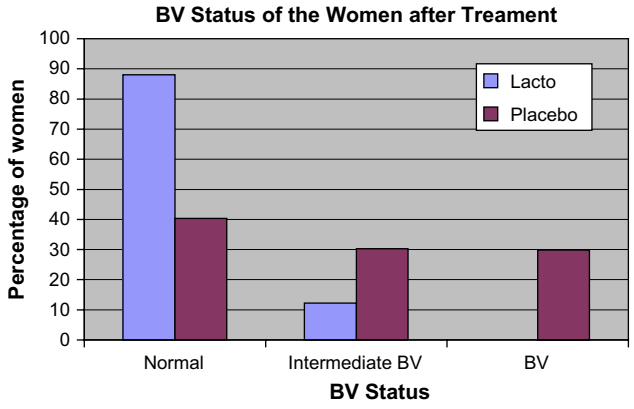

Fig. 1. Percentage of women with BV status after treatment as determined by Nugent scoring and BVBlue test. Lacto = group of 49 women treated with metronidazole and probiotic *Lactobacillus* strains GR-1 and RC-14. Placebo = group of 57 women treated with metronidazole and placebo. $p < 0.001$ for comparison of 88 versus 40% normal groups.

4. Discussion

This study demonstrated that oral probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 treatment augmented the efficacious treatment of BV with metronidazole. A number of studies have assessed various antibiotic protocols for the treatment of BV in Caucasian, African-American, European, Asian and Mexican women, with failure rates as high as 39% [13–18]. Twice daily 500 mg metronidazole for 7 days is a standard treatment for BV, but no study on its use in African women has been reported. Thus, it is difficult to assess why only 40% of subjects receiving antibiotic and placebo were cured of the condition. One explanation could be that BV is more difficult to eradicate in Nigerian women, for whatever reason. Under such a scenario, the 88% cure with antimicrobial drug and probiotic is particularly impressive. If intermediate Nugent and sialidase findings are included as cures, then the failure rate for antibiotic/placebo is 30%, which is more consistent with the literature. However, under that scenario, the antibiotic/probiotic success rate would be 100%, again 10–39% higher than most studies with antibiotic alone [19].

This study was not designed to understand the mechanisms of action of the therapies. However, it is known that antibiotics function by killing the pathogens, while *Lactobacillus* GR-1 and RC-14 are known to inhibit urogenital pathogen growth and adhesion [20], displace BV organisms [11], downregulate vaginal inflammation and enhance immune defenses (Kirjavainen et al., submitted for publication), all of which could explain the effectiveness of the combined treatment used in this study. The concept of replenishing the vaginal microbiota with exogenous probiotic lactobacilli has been considered for the past 25 or so years. Under normal circumstances, the patient's own lactobacilli would return after antibiotic therapy and colonize the vagina, thereby conferring some protection from infection, as seen in the placebo group here. The additional use of probiotics is designed to enhance this process, and as noted in the present study, this leads to improvements in disease management and recovery.

Only one previous study has tested the efficacy of probiotics combined with antibiotics for BV. Although not published in peer-reviewed literature, the company that performed the study reported that treatment with *Lactobacillus crispatus* CTV-05 resulted in vaginal colonization in 62% of patients at 30 days and satisfactory clinical endpoint was found in 50% of patients in probiotic and placebo groups (http://www.themedicinescompany.com). By comparison, the ability of *Lactobacillus* GR-1 and RC-14 to colonize the vagina after oral intake [6,7] and the complete recovery of a lactobacilli microbiota coinciding with BV cure that resulted here, demonstrate the merits of this strain combination along with 7-day metronidazole therapy.

The fact that this study was undertaken on African women, known to have relatively high rates of BV and to be at high risk of HIV, is significant. HIV infection rates are skyrocketing amongst women in sub-Saharan Africa, with over 7000 new cases reported each day. It is not known if the correlation between BV and HIV is due to elevated inflammation, loss of lactobacilli or some other host affinity change, but it seems logical to test whether eradication of BV can reduce the risk of HIV. The current findings, particularly if verified by a second, clinical trial, provide strong support for testing probiotic use in women at high risk of HIV. Metronidazole is now genericized and is relatively inexpensive. It does not generate the resistance problems reported for clindamycin [21]; thus it provides a good antimicrobial option for combined use with probiotic lactobacilli for management of BV.

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