Advance Publication by J-STAGE

Japanese Journal of Infectious Diseases

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Received: May 23, 2019. Accepted: December 5, 2019
Published online: December 25, 2019
DOI:10.7883/yoken.JJID.2019.190

Advance Publication articles have been accepted by JJID but have not been copyedited or formatted for publication.
Relationship between Human β-defensin 2 and the vaginal environment

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Keywords: bacterial vaginosis, human β-defensin 2, vaginal Candidiasis, vaginal environment

Running head: Human β-defensin 2 in vagina
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Summary

As one of the main antimicrobial peptides, human β-defensin 2 (HBD2) plays multiple roles in the lower genital tract. Based on the Nugent score as a diagnostic criterion for bacterial vaginosis, we sought to clarify the correlations among the Nugent score and Interleukin-6 (IL-6) and HBD2 levels in vaginal secretions in association with various types of infection. Ninety-eight women were recruited for this study. Levels of HBD2 and IL-6 in the vaginal wash were measured by enzyme-linked immunosorbent assays. According to the Nugent method, the number of Lactobacillus morphotypes per field of view was well correlated with the HBD2 level. The amount of HBD2 was also well correlated with the presence of Candida spp. (p<0.01). In vitro experiments revealed that the expression of HBD2 from the human vaginal epithelial cell line, VK2/E6E7, was induced by the addition of heat-killed Candida albicans (HKCA). The addition of HKCA induced the expression of Dectin-1 mRNA, and a luciferase assay for NF-kB responsive elements showed that HKCA activated NF-kB signaling. These results suggested that Candida albicans would be induced the activation of Dectin-1 and NF-kB signaling, resulting in HBD2 expression. In conclusion, the expression of HBD2 was positively correlated with the presence of Lactobacillus and Candida spp.
Introduction

Commensal bacteria in the vagina represent one of the first line of defense against vaginal infection. Some *Lactobacillus* species, in particular, play important roles in this defense (1, 2). The bacterial population in the vaginal flora is altered prior to the presentation of symptoms of vaginosis. Therefore, the Nugent score was developed for the diagnosis of bacterial vaginosis (BV) (3). However, there have been some reports that the Nugent score doesn’t always reflect the symptoms or degree of infection (4-7), as the vaginal environment is maintained by lactic acid-producing bacteria even in the absence of *Lactobacillus* species in most healthy women. In addition, dominant *Lactobacillus* species seems to differ across race and cultural divides (8). Therefore, the diagnosis of BV based on the Nugent score alone remains problematic and is likely to lead to unnecessary medical treatment and disturbance of the vaginal flora.

Innate immunity is important to the protection of the lower genital tract from bacterial, fungal and viral infections. Antimicrobial peptides play multiple roles in the innate immune mechanisms, including chemotaxis, induction of cytokines and wound healing (9). Antimicrobial peptides are composed of less than 100 amino acids and have many positively charged residues and an amphipathic structure (9). Although the precise mechanism underlying their antimicrobial action remains to be elucidated, antimicrobial peptides are predicted to make a pore in the target membrane through which to exhibit their antimicrobial activity (10).

Humans and other mammals possess two main families of antimicrobial peptides; defensins and cathelicidins (11). Defensins are divided into three main subfamilies; the α-, β- and θ-defensins, which differ in terms of the length of the peptide segments between the cysteine residues connected by disulfide bonds (11).
Human β-defensins (HBDs) consist of four species; HBD1, HBD2, HBD3, and HBD4. Among them, HBD1–3 are expressed in vaginal epithelium. HBD1 is constitutively expressed in epithelial cells, whereas HBD2 and 3 are induced by the infection of various microbes and inflammation (12-14). These signals were shown to activate transcription factors, NF-kB and AP-1, thereby inducing the expression of HBD2 and HBD3 in *in vitro* experiments (15, 16). However vaginal HBD2 level was lower in women with BV than without BV, HBD2 is involved in the innate immunity in the vagina through its antimicrobial activity against *Escherichia coli*, *Pseudomonas aureginosa*, *Candida albicans* (17, 18). On the other hand, β-defensin is increased in the vaginal fluid of an animal Candidiasis model (19). In addition, HBD1 and HBD2 show chemotactic activity for T cells and immature dendritic cells through a chemokine receptor, CCR6 (20). In this study, we examined the correlation between the Nugent score and HBD2 levels and in association with various types of infection as a biochemical marker for evaluation of the vaginal environment.

**Materials and Methods**

**Participants and sample collection**

Ninety-eight non-pregnant women attending Ehime University Hospital or NTT West Matsuyama Hospital were recruited for this study from July through October, 2012. The background characteristics are summarized in Table 1. Exclusion criteria included malignant diseases, autoimmune diseases, inflammatory bowel diseases, and antibiotics or steroid hormone use. Informed consent in writing was obtained from all participants prior to enrolment in the study, and the study was approved by the Ethics Committee of Ehime University Hospital (Permission No: 1206212). Vaginal secretions
were collected using cotton swabs for the calculation of the Nugent score. The vagina was subsequently washed with 4 mL of saline, which was collected for bacterial analysis. A portion of the vaginal wash was centrifuged at 3,000 rpm for 10 min at 4°C and the supernatant was stored at -80°C until use for IL-6 and HBD2 assays. All experimental methods were performed in accordance with the approved guidelines.

**Terminal restriction fragment length polymorphism (t-RFLP) and microbial analyses by polymerase chain reaction (PCR)**

DNA was extracted from the vaginal wash using a QIAamp DNA mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. The DNA was then amplified by PCR using the following primer set: LYS (5’-FAM-AGAGTTTGATCCTGGCTCAG-3’; the 5’-end was labeled with FAM) and LYSrev [5’-CCGTCAATTC(A/C)TTT(A/G)AGTTT-3’] (Applied Biosystems, Waltham, MA). PCR reactions were carried out using the standard protocol at a volume of 25 μL containing 0.4 mM of each dNTP (TaKaRa Bio, Shiga, Japan), 2.0 mM of MgCl₂, 0.15 μM of each primer and 0.5 U KOD FX Polymerase (Toyobo, Osaka, Japan). The reaction profile was as follows: after initial denaturation at 94 °C for 2 min, DNA was amplified with 35 cycles of 98°C for 10 s, 60 °C for 30 s, and 72 °C for 70 s, and then extended at 72 °C for 7 min. PCR products were digested with the Msp I restriction enzyme (TaKaRa Bio) and 1 μl of digested product was combined with 9 μl of Hi-Di formamide (Applied Biosystems) and 1 μl of GeneScan 120 LIS Size standard (Applied Biosystems). The samples were analyzed with an ABI PRISM 3100 Genetic Analyzer using GeneScan analysis software version 3.7 (Applied Biosystems). The t-RFLP score was determined by the number of peaks as follows: low = less than 3 peaks,
intermediate = 3 to 5 peaks, and high = 6 peaks or more. Gram-stained vaginal fluid smears were examined under an oil immersion objective lens at 1000 x magnification. In the Nugent scoring method, the presence of large Gram-positive rods (*Lactobacillus* morphotype), small Gram-variable rods (*Gardnerella* morphotype), and curved Gram-negative or -variable rods (*Mobiluncus* morphotype) were quantified. The composite score was categorized into one of the three groups. Scores of 0 to 3 were considered normal (*Lactobacillus* dominant), 4 to 6 were labeled as intermediate (mixed morphotype), and 7 to 10 were BV (absence of *Lactobacilli* and predominance of *Gardnerella* and *Mobiluncus* morphotype). We determined the presence of *Candida* spp. in vaginal flora by microbial cultivation and microscopic analysis.

Furthermore, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium*, and *Chlamydia trachomatis* were analyzed using PCR. Oligonucleotide primers for the PCR reactions were designed as shown in Table 2 and the PCR reactions was carried out using EmeraldAmp PCR Master Mix (TaKaRa Bio) with a Thermal Cycler (Chromo4 System, Bio-Rad Laboratories, Hercules, CA). The reaction profile was as follows: initial denaturation at 94 °C for 2 min, 35 cycles of 98 °C for 10 s, 54 °C for 30 s, and 72 °C for 45 s, and then a final extension at 72 °C for 7 min.

**HBD2 and IL-6 ELISA**

The HBD2 and IL-6 levels in the vaginal wash were measured by enzyme-linked immunosorbent assays (ELISA; hBD2 ELISA kit: Phenix Pharmaceuticals, Inc., Mannheim, Germany; IL-6 Quantikine ELISA kit: R&D Systems, Inc., Minneapolis, MN) according to the manufacturers’ instructions. Light absorbance was measured at 450 nm on a microplate reader (iMark; Bio-Rad Laboratories). The
Cell culture

A human vaginal epithelial cell line (VK2/E6E7; CRL-2616, American Type Culture Collection, Manassas, VA, USA) was cultivated in keratinocyte serum-free medium (KSFM; Thermo Fisher Scientific, Waltham, MA) supplemented with 50 µg/ml of bovine pituitary extract, 0.1 ng/ml of recombinant epidermal growth factor, 0.4 mM CaCl2 and antibiotics including penicillin, streptomycin and amphotericin (Sigma-Aldrich, St. Louis, MO). The VK2/E6E7 cells were seeded in a 24-well plate at 1 x 10^5 cells per well and the culture was incubated with a final concentration of 10 µg/ml LPS (Sigma-Aldrich, St. Louis, MO) or a multiplicity of infection (MOI) of 10 cfu per cell of a heat-killed preparation of *Candida albicans* (HKCA; Thermo Fisher Scientific; final concentration) in KSFM for 24 h at 37°C. The supernatant was retrieved and centrifuged at 600 rpm for 5 min. The aliquot was stored at -80 ºC until measurement of HBD2.

Determination of gene-expressions by RT-PCR

After 24 hours from stimulation by HKCA, the expression of mRNAs from Dectin-1, Dectin-2, Mincle and Mannose receptor genes in the VK2/E6E7 cells were analyzed by reverse transcription PCR (RT-PCR). Total RNA was extracted from the cells by TRIzol reagent (Thermo Fisher Scientific) and RNeasy Mini Kit (Qiagen), and reverse transcribed into complementary DNA (cDNA) using an iScript cDNA synthesis kit (Bio-Rad Laboratories) in accordance with the manufacturer’s instructions. Oligonucleotide primers were designed as shown in Table 2. RT-PCR was performed
with iQSYBR Green Supermix (Roche Diagnostics, Basel, Switzerland) using a Chromo4 Thermal Cycler (Bio-Rad Laboratories). PCR consisted of an initial denaturation step of 2 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C, and a final extension of 15 min at 72 °C. Semi-quantitative analysis was performed by correction against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The mRNA of TLRs were detected by TLR RT-Primer set (InvivoGen).

Luciferase assay

The VK2/E6E7 cells were transfected with a reporter plasmid (pGL4.32) containing NF-kB responsive elements at the luciferase promoter region using the Gene Pulser II electroporation system (Bio-Rad Laboratories). In brief, 40 μg of the plasmid was added to a trypsinized cell suspension (1x10^7 cells) in a 0.4 mm cuvette. After electroporation (260 V, 975 μF, ∞ Ω), DMEM containing 20% fetal calf serum but no antibiotics was added to the cuvette, and the suspension was transferred to 35-mm dishes. After culturing for 24 h, the culture medium was changed to KSFM containing 1x10^8 cells/ml of HKCA. After culturing for a further 24 h, the cells were harvested and analyzed with a luciferase detection kit (TOYOBO). The assay was repeated in triplicate.

Immunofluorescent assay

The VK2/E6E7 cells were grown on coverslips. Cells were fixed with 4% paraformaldehyde, permeabilized with or without 0.05% Triton X-100, and subjected to an immunofluorescence assay using with DAPI, anti-Dectin1 rat mAb (2A11; Abcam, Cambridge, England) and anti-Rat IgG-Alexa 488 antibody (Thermofisher Scientific).
Samples were analyzed with Carl Zeiss LSM 700 confocal microscope system (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All statistical analyses were performed using EZR software (21) (Saitama Medical Center, Jichi Medical University, Saitama, JAPAN). Data are expressed as the average [range] or mean ± SD. Other experiments on human specimens were analyzed by Mann-Whitney U test. RT-PCR results were analyzed using the Mann-Whitney U test. Differences were considered to be significant at $P < 0.05$.

Results

Correlations among Nugent Score and t-RFLP, HBD2 and IL-6 levels

Vaginal swab specimens were analyzed by Gram-staining according to the Nugent criteria. The prevalence of normal, intermediate, and BV was 60.2%, 22.4%, and 17.3%, respectively (Table 1). The t-RFLP profiles were classified into three groups based on the number of bands as described in Materials and Methods. According to our analysis, the t-RFLP profiles were highly correlated with the Nugent score with a kappa value of 0.76. There was no statistically significant correlation between the Nugent Score and HBD2. However, the Nugent score for Lactobacillus morphotype point was well correlated with HBD2. The higher Lactobacillus morphotype group was 0-2 points and the lower group was 3-4 points (Table 3). IL-6, a marker of inflammation, was not correlated with the Nugent Score or the Lactobacillus morphotype point.

In addition, we determined the relationships between the presence of Candida spp., Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum, and
Chlamydia trachomatis in the vaginal flora and the results of the ELISA analysis. As shown in Table 4, the HBD2 level was only well correlated with the presence of Candida spp. Vaginal candidiasis was treated with an oxiconazole nitrate vaginal tablet 600mg. After anti-fungal treatment, the HBD2 level was decreased in Candidiasis patients (Fig.1).

The induction of HBD2 via NF-kB signaling in vaginal epithelial cells

HBD2 is known to be produced by epithelial cells. We evaluated the production of HBD2 from the vaginal epithelial cell line VK2/E6E7 cells. After stimulation by HKCA or LPS for 24 hr, the supernatant of the VK2/E6E7 cell culture was analyzed using ELISA. As shown in Figure 2A, the production of HBD2 was stimulated by HKCA but not by LPS. TLR4 mRNA was not detected in VK2/E6E7 cells (data not shown). As β-glucan Candida albicans were previously reported to be recognized by the Dectin-1 receptor (22, 23), we analyzed the expression of Dectin-1 mRNA in comparison with Dectin-2, Mincle, and Mannose receptor mRNAs in VK2/E6E7 cells exposed to HKCA. Only Dectin-1 mRNA expression was enhanced by HKCA (Fig. 2B). The protein expression of Dectin-1 in VK2/E6E7 cells was confirmed with immunofluorescence assay (Fig. 2C). In addition, luciferase assay demonstrated the induction of NF-kB signaling activity in the presence of $10^8$ cells/ml of HKCA (Fig. 2D).

Discussion

In the present study, we compared the Nugent score and HBD2 level in vaginal fluid. Although the HBD2 level was not correlated with Nugent score or IL-6, it was
correlated with the presence of *Lactobacillus* (*Lactobacillus* morphotype point). *Lactobacillus* species in general and *L. crispatus* in particular, which is known as a “good bacteria,” were previously reported to induce the expression of HBD2 in cultured cells (24). The high level of HBD2 induced by *L. crispatus* is thought to prevent the growth of some Gram-negative bacteria and the pathogenesis of BV (9). Also, there is the report that HBD2 production is stimulated in the vaginal epithelium cells by *Lactobacillus jensenii* (25). Nugent score is based on the presence or absence of *Lactobacillus*, but also depend on present of bacteria such as *Mobiluncus, Gardnerella.* According to our analysis, Nugent score was highly correlated with the t-RFLP score. Therefore it is clear that Nugent score reflects real bacteria. We showed that HBD2 reflected increase of *Lactobacillus* than a state of bacteria.

Although IL-6 is a marker of inflammation, our results indicated that there were no statistically significant association between IL-6 and clinical criteria of BV, as shown in Table 3. The relationship between BV and IL-6 has been reported in many studies with various results. Mitchell *et al.* reported a relationship between BV and decreased HBD2 expression but no relation to IL-6 (26). On the other hand in *Chlamydia trachomatis* infection, IL-6 is reportedly increased with BV patients but not with *Lactobacillus* dominant flora (27). Our results suggested that IL-6 might not be strongly involved in the pathogenesis of BV and Candidiasis in some vaginal environments (Table 3 and 4).

We also showed that vaginal HBD2 level was increased in Candidiasis but not in other microbes. HBD2 would be increased by the overgrowth of *Candida spp.*, because the HBD2 level of patients was reduced by the anti-fungi treatment (Fig. 1). Most of Candidiasis patients presented normal Nugent score and higher *Lactobacillus*
morphotype point, because *Candida* are able to cohabitate with *Lactobacillus*. In this study, 69% of candida patients were the normal of Nugent score and normal of *Lactobacillus* morphotype point. It is reported that *Lactobacillus* could inhibit the overgrowth of *Candida albicans* by producing a wide variety of secondary metabolites with antimicrobial activity, such as lactic acid, hydrogen peroxide, bacteriocin-like compounds, and biosurfactants (28). In our study, HBD2 was increased in vaginal fluid of Candidiasis patients who had highly *Lactobacillus* morphotypes. The cohabitait of *Candida* and *Lactobacillus* would induce the expression of HBD2.

We showed HBD2 increases under the presence of *Candida* spp. and is found to respond for *Candida* sterilization by anti-fungal treatment (Fig. 1). Therefore we examined it in vitro, production of HBD2 was significantly increased by the HKCA stimulation on the vaginal epithelial cell line VK2/E6E7 cells (Fig. 2A). Fungal species are recognized by immune system via pattern recognition receptors (PRRs) such as Toll-like receptor (TLR) family and C-type lectin receptors (CLRs) (29). CLRs pathway are critical for immune responses to fungal species (30). Previous studies demonstrated that CLRs, mainly Dectin-1 and Dectin-2 play important roles in host immune response to *Candida albicans* (31, 32). Some studies reported Dectin-1 and Dectin-2 are expressed on other epithelial cell line cells, pulmonary epithelial cells (33), corneal epithelial cells (34), intestinal epithelial cells (35). We demonstrated that the expression of Dectin-1 in VK2/E6E7 cells and the activation of NF-kB signaling by the addition of HKCA (Fig. 2). Although other receptors might be involved, Dectin-1 is the candidate receptor for this signaling because Dectin-1 is able to recognize β-1,3-glucan which is derived from the cell walls of several fungi such as *Candida, Aspergillus* and many other fungal species (36). The production of HBD2 is stimulated by microbial materials
through NF-kB signaling activation (16, 37). Therefore the activation of NF-kB through Dectin-1 would lead to induce the expression of HBD2.

In conclusion, HBD2 was an important factor for the barrier against the infection of microbes, and it was positively correlated with the presence of Lactobacillus and Candida spp. as shown by the results of the present study.

Acknowledgments

This work was partially supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant No: MO17H04346) and a grant for Research on Child Development and Diseases from AMED (Grant No: JU29922).

Conflict of Interest

We declare that we have no conflicts of interest.

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Figure legends

Fig. 1. Concentration of HBD2 before and after treatment for vaginal candidiasis
After anti-fungal treatment, the HBD2 level was significantly decreased in Candidiasis patients.
Data were expressed as the median [inter-quartile range].
* P <0.05

Fig. 2. Production of HBD2 through Dectin-1
A. Production of HBD2 by VK2/E6E7 cells was measured by ELISA. HBD2 level was significantly higher after stimulation by HKCA but not by LPS. Data were expressed as the median [inter-quartile range].

B. After 24 hours from stimulation by HKCA, the expression of receptor mRNAs in VK2/E6E7 were analyzed by RT-PCR. HKCA induced the expression of Dectin-1 but not the Dectin-2, Mincle or Mannose receptor. Data are expressed as the mean ± SD.

C. The expression of Dectin-1 was analyzed with immunofluorescent assay. After treated with (a) or without (b) 0.05% Triton-X100, VK2/E6E7 cells were stained
with anti-Dectin1 antibody (Green) and DAPI (Blue) as described in Materials and Methods.

D. Luciferase assay of VK2/E6E7 cells transfected with pGL4.32 containing NF-kB responsive elements. It demonstrated the induction of NF-kB signaling activity in the presence of $10^8$ cells/ml of HKCA. Data are expressed in relative luminescence units (RLU) as the mean ± SD. * $P < 0.05$, ** $P < 0.01$
### TABLE 1
Demographic and clinical information at enrollment

| Characteristic                      | n=98         |
|-------------------------------------|--------------|
| Age, mean ± SD (min-max)            | 39 ± 10 y.o. (20-65) |
| 20-29                               | 20 (20%)    |
| 30-39                               | 34 (35%)    |
| 40-49                               | 33 (34%)    |
| 50-59                               | 8 (8%)      |
| 60-                                 | 3 (3%)      |
| Gravidity, mean (min-max)           | 1.2 (0-4)   |
| Primigravida                         | 41 (42%)    |
| Multigravida                         | 57 (58%)    |
| Parity, mean (min-max)              | 1.0 (0-3)   |
| Primipara                           | 46 (47%)    |
| Multipara                           | 52 (53%)    |
| Pre-menopausal                      | 90 (91%)    |
| Post-menopausal                     | 8 (8%)      |
| Smoker                              | 19 (19%)    |
| Nugent score                        |             |
| normal (0 – 3)                      | 59 (60.2%)  |
| intermediate (4 – 6)                | 22 (22.4%)  |
| BV (7 – 10)                         | 17 (17.3%)  |

BV: bacterial vaginosis

Ninety-eight non-pregnant women were recruited for this study. The mean age of them was 39 ± 10 years old and was mean of 1.2 gravida 1.0 multipara. The prevalence of normal, intermediate, and BV was 60.2%, 22.4%, and 17.3%, respectively.
| Primer                                      | Sequence (5’ -> 3’)                      |
|---------------------------------------------|------------------------------------------|
| Ureaplasma ulealyticum U-5F                | CAATCTGCTCGTGGAAGTATTAC                  |
| Ureaplasma ulealyticum U-4R                | ACGACGTCCATAAGCAACT                     |
| Mycoplasma genitalium MgPaW1F              | AAGTGGAGCGATCATTACTAACC                 |
| Mycoplasma genitalium MgPaR1R              | CCGTTGTATCATACCTTTTCTGA                 |
| Mycoplasma hominis F-P                     | GACACTAGAAACTAGAGTTAG                   |
| Mycoplasma hominis R                       | CACCATCTGTCACTCTGTTAACCCTC             |
| 1st PCR Chlamydia trachomatis             | TTGCGATCCTTGCAACCACCTT                  |
| Chlamydia trachomatis                     | GCTCGAGACCATTAACTCC                     |
| Nested PCR Chlamydia trachomatis          | GGTAACCTTTGTTTTCGACCG                   |
| Chlamydia trachomatis                     | CTCCAATGTAGGGAGTGAA                     |
| Dectin-1 forward                           | TGGGAGGATGGATCAACATT                    |
| Dectin-1 reverse                           | TGGGTTTTCTTGGGTAGCT                     |
| Dectin-2 forward                           | TTCAGTGAGGGACAAAGGTG                    |
| Dectin-2 reverse                           | AAGTAGCAACTGGAAACAAATGGA                |
| Mincle forward                             | CTGGGATCCCCATCCTATTT                    |
| Mincle reverse                             | TTTGAAAGATGCGAAATGTCA                   |
| Mannose Receptor forward                   | CACCATCGAGGAATTGGACT                    |
| Mannose Receptor reverse                   | ACAATTCGTCATTGTGCTCA                    |
TABLE 3

Concentrations of HBD2 and IL-6 in the vaginal fluid from women with and without BV

| Lactobacillus morphotype point | n   | Age  | HBD2 (ng/ml)          | IL-6 (pg/ml)   |
|-------------------------------|-----|------|-----------------------|---------------|
| normal (0-3)                  | 59  | 38 ± 8 | 28.8 [9.7-87.2]       | 30.5 [5.5-81.4] |
| intermediate (4-6)            | 22  | 41 ± 11 | 39.6 [16.7-79.8]      | 20.0 [9.0-84.8] |
| BV (7-10)                     | 17  | 36 ± 12 | 32.9 [6.2-56.8]       | 23.1 [7.3-47.9] |
| 0-2 (≥1/oil immersion field)  | 66  | 38 ± 8 | 39.2 [14.9-97.4]**    | 31.6 [6.1-99.3] |
| 3-4 (<1/oil immersion field)  | 32  | 40 ± 12 | 19.0 [5.1-48.1]**     | 18.8 [4.6-63.9] |

**P<0.01

HBD2: human beta defensin 2, IL-6: interleukin 6, BV: bacterial vaginosis

There was no statistically significant correlation between the Nugent score and HBD2. The Lactobacillus morphotype point was well correlated with HBD2 (P<0.01). IL-6 was not correlated with Nugent score or the Lactobacillus morphotype point. Data were expressed as the median [inter-quartile range].
**TABLE 4**
Concentrations of HBD2 and IL-6 in the vaginal fluid from women with and without microbes

| Group                  | n  | HBD2 (ng/ml)     | IL-6 (pg/ml)   |
|------------------------|----|------------------|----------------|
| **Candida spp.**       |    |                  |                |
| (+)                    | 13 | 92.7 [48.4-215.8]* | 52.3 [16.1-79.9]|
| (-)                    | 85 | 28.9 [9.5-61.7]*  | 23.1 [3.5-81.7]|
| **Mycoplasma hominis** |    |                  |                |
| (+)                    | 7  | 37.1 [8.2-108.4]  | 31.7 [14.6-52.2]|
| (-)                    | 91 | 30.7 [9.7-77.6]   | 23.3 [5.6-81.7]|
| **Mycoplasma genitalium** |  |                  |                |
| (+)                    | 4  | 28.3 [8.3-48.6]   | 36.8 [11.1-76.0]|
| (-)                    | 94 | 31.8 [10.1-82.9]  | 25.0 [5.0-81.6]|
| **Ureaplasma urealyticum** | |                  |                |
| (+)                    | 40 | 35.0 [8.7-101.4]  | 30.3 [12.0-81.3]|
| (-)                    | 58 | 30.0 [10.1-61.3]  | 15.4 [3.4-85.6]|
| **Chlamydia trachomatis** |  |                  |                |
| (+)                    | 5  | 23.7 [16.0-32.9]  | 25.5 [23.3-29.1]|
| (-)                    | 93 | 33.1 [9.5-81.8]   | 24.5 [4.6-81.7]|

*P <0.05

HBD2: human beta defensin 2, IL-6: interleukin 6, PCR: polymerase chain reaction, t-RFLP: terminal restriction fragment length polymorphism

The HBD2 levels were only correlated with the presence of *Candida spp.* Other microbes did not correlate with it. Data were expressed as the median [inter-quartile range].
FIGURE 1
Concentration of HBD2 before and after treatment about vaginal candidiasis
FIGURE 2  Production of HBD2 through Dectin-1

A

B

C

D

![Box plot showing HBD2 levels in control, HKCA, and LPS treated samples.](image)

![Bar graph showing fold change in HBD2 production.](image)

![Images of cell staining for a and b.](image)

![Bar graph showing RLU fold change with different concentrations of HKCA.](image)