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Cervicovaginal Levels of Human β-Defensin 1, 2, 3, and 4 of Reproductive-Aged Women With *Chlamydia trachomatis* Infection

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Objective: This study included women attending primary health care units in Botucatu, São Paulo, Brazil, to assess the cervicovaginal levels of human β-defensin (hBD) 1, 2, 3, and 4 during *Chlamydia trachomatis* infection.

Patients and Methods: Cervicovaginal samples were collected for Pap testing and assessing the presence of infection by *C. trachomatis,* human papillomavirus, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. Vaginal smears were taken to evaluate local microbiota. Human β-defensin levels were determined using enzyme-linked immunosorbent assay in cervicovaginal fluid samples. Seventy-four women with normal vaginal microbiota and no evidence of infection were included in hBD quantification assays; 37 tested positive for *C. trachomatis* and 37 were negative. Statistical analysis was performed using Mann-Whitney *U* test.

Results: Women positive for *C. trachomatis* had significantly lower cervicovaginal hBD-1, hBD-2, and hBD-3 compared with those who tested negative (hBD-1: 0 pg/mL [0–2.1] vs 1.6 pg/mL [0–2.4], $p < .0001$; hBD-2: 0 pg/mL [0–3.9] vs 0.61 pg/mL [0–8.9], $p = .0097$; and hBD-3: 0 pg/mL [0–4.3] vs 0.28 pg/mL [0–8.4], $p = .0076$). Human β-defensin 4 was not detected.

Conclusions: Lower levels of hBD-1, hBD-2, and hBD-3 in cervicovaginal fluid were detected in the presence of *C. trachomatis* infection.

Key Words: human β defensins, *Chlamydia trachomatis*, antimicrobial peptides, cervicovaginal fluid, women

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Chlamydia trachomatis is the most common sexually transmitted bacteria, and an estimated global prevalence of *C. trachomatis* infection in women of 4.2% (131 million cases) was reported in 2012.[1]

The most frequent manifestation of *C. trachomatis* infection is a purulent endocervicitis, although it often remains asymptomatic. Lack of signs of infection are due to low bacterial pathogenicity, which involves a bacterial wall composed of lipopolysaccharide with reduced potential for host immune system activation[2] and ability of the microbe to persist intracellularly.[3] The development of endocervicitis is not exclusively dependent on bacterial pathogenicity but also on the host's immune system activity and the production of factors that may inhibit this pathogen's growth.[4]

Elimination of *C. trachomatis* infection by the host requires activation of innate and adaptive immune responses.[2,4] Humans and animals infected with *C. trachomatis* produce interferon γ (IFN-γ),[5,6] which controls bacterial replication by stimulating macrophage phagocytic potential, promoting elimination of this pathogen via phagocytosis. Interferon γ also inhibits infected cell growth by inducing nitric oxide synthase expression that destroys intracellular bacteria.[7] Although some individuals clear their infection due to an effective Th1/IFN-γ response, others develop chronic infections or are prone to repeated infections.

Antimicrobial peptides, such as human defensins, participate in the primary host defense against microorganisms. Defensins are cationic molecules that are attracted to the anionic surface of the microorganism membrane[8] and the defensin-pathogen interaction generates an electrostatic imbalance that forms pores in the microbial membrane, loss of selective permeability, and microorganism lysis.[9,10] Defensins may also act indirectly by inducing intracellular hydrolases, which alter the pathogen cell wall and interfere with membrane lipid distribution.[10]

Human β-defensins (hBDs) 1, 2, 3, and 4 represent the main group of human antimicrobial peptides and are produced by epithelial and immune cells in response to infection.[11] The expression of hBD-1 is constitutive; however, its biosynthesis is increased in the presence of inflammation or lipopolysaccharide.[12,13] Human β-defensin 2 acts mainly on gram-positive bacteria and is regulated through nuclear factor κ B (NF-κB) transcription factor.[14] A wider spectrum of action is shown by hBD-3, which is also more resistant to the higher salt concentration in the physiological environment.[9,15] Human β-defensin 2 regulation is achieved by AP-1 transcription factor, formed upon mitogen-activated protein kinase triggered through epidermal growth factor receptor.[16]

Considering the important role played by β-defensins in innate immunity response to microorganisms, this study aimed to determine the cervicovaginal levels of hBD-1, hBD-2, hBD-3, and hBD-4 in reproductive-aged women with *C. trachomatis* infection.

PATIENTS AND METHODS

Study Groups

This study involved 37 reproductive-aged women with *C. trachomatis* infection and 37 without infection, attended at primary health care units in Botucatu, São Paulo, Brazil, from September 2012 to January 2013. All the women presented normal vaginal flora, no cervical lesions, and no human papillomavirus (HPV), *Trichomonas vaginalis,* or *Neisseria gonorrhoeae* infection at the time of enrollment. The eligibility criteria included women who were nonpregnant, nonmenopausal, had no

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The authors declare there are no conflicts of interest.

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This study was approved by the local Research Ethics Committee (UNESP), under protocol 4121-2012.

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TABLE 1. Sociodemographic Characteristics, Sexual Behavior, and Clinical History of the Reproductive-Aged Women Included in the Study

Variables	C. *trachomatis* negative	C. *trachomatis* positive	*p*
Age,[a] (years)	33 (19–50)	32 (18–48)	.67
Marital status,[b] *n* (%)			
Single	8 (21.6)	16 (43.2)	.08
Married	29 (78.4)	21 (56.8)	
Race,[b] *n* (%)			
White	22 (59.4)	25 (67.6)	.62
Nonwhite	15 (40.6)	12 (32.4)	
Education,[b] *n* (%), y			
≤9	14 (37.8)	14 (37.8)	.74
≥10	18 (48.6)	20 (54)	
≥15	5 (13.6)	3 (8.2)	
Sexually active,[b] *n* (%)			
Yes	35 (94.6)	29 (78.4)	.08
No	2 (5.4)	8 (21.6)	
Lifetime partners[a]	2 (1–30)	1 (1–30)	.72

[a]Nonparametric Mann-Whitney *U* test.
[b]χ^2 test.

hysterectomy, no previous report of HIV seroconversion, no vaginal bleeding, no urinary loss, no antibiotics or vaginal cream use in the preceding 30 days, and abstinence from sexual intercourse in the 72 hours preceding the visit.

Samples

Sociodemographic, sexual behavior, and gynecological history data were obtained by interview. Then, the women underwent a gynecological examination, and cervical samples were collected for molecular analysis of HPV, *C. trachomatis*, and *N. gonorrhoeae.* Midvaginal wall samples were collected using a cotton swab to evaluate the vaginal microbiota pattern according to Nugent criteria[17] and from the posterior fornix of the vagina to assess infection by *T. vaginalis*. Lastly, 1 mL of cervicovaginal fluid was collected to determine hBD-1, hBD-2, hBD-3, and hBD-4 levels by enzyme-linked immunosorbent assay. Samples were stored at −80ºC until processing.

All the women enrolled signed a term of written consent, and the study was approved by the local research ethics committee (Universidade Estadual Paulista), under protocol 4121-2012.

Human Papillomavirus, *C. Trachomatis, N. Gonorrhoeae,* and *T. vaginalis* Detection

DNA from endocervical samples was extracted using the AmpliLute Liquid Media Extraction kit (Roche Molecular Systems, Inc), following the manufacturer's instructions. The human β-globin target was co-amplified to determine sample adequacy. *Chlamydia trachomatis* and *N. gonorrhoeae* were detected by polymerase chain reaction, as previously described.[18,19] Infection by *T. vaginalis* was examined by culture in Diamond medium at 37ºC.

Quantification of Cervicovaginal Levels of β-Defensins

Cervicovaginal fluid samples were evaluated by enzyme-linked immunosorbent assay using Peprotech-specific kits (Rocky Hill, NJ) to quantify hBD-1 (cat#900-M202), hBD-2 (cat#900-M172), hBD-3 (cat#900-M210), and hBD-4 (cat#900-M435), following the manufacturer's instructions. Minimum detectable levels were 0.05, 0.13, and 0.01 pg/mL for hBD-1, hBD-2, and hBD-3, respectively.

Statistical Analysis

Data obtained were analyzed by the Mann-Whitney *U* test or the χ^2 test. Results with a *p* value of less than .05 were considered statistically significant. Statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA).

Role of the Funding Source

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RESULTS

The sociodemographic characteristics, sexual behavior, and clinical history of the participating reproductive-aged women are shown in Table 1. No significant differences were observed between the groups (see Table 1).

The results of cervicovaginal β-defensin quantification showed that a large number of samples had detectable levels of hBD-1 (58.1%), hBD-2 (29.7%), and hBD-3 (43.2%), regardless of *C. trachomatis* status. In contrast, levels of hBD-4 remained below the minimal detection limit of the assay in all samples.

Figure 1 shows the cervicovaginal levels determined for hBD-1, hBD-2, and hBD-3 regarding *C. trachomatis* infection status. Significantly lower levels of hBD-1 (0 pg/mL [0–2.1] vs 1.6 pg/mL [0–2.4], $p < .0001$), hBD-2 (0 pg/mL [0–3.9] vs 0.61 pg/mL [0–8.9], $p = .0097$), and hBD-3 (0 pg/mL [0–4.3] vs 0.28 pg/mL [0–8.4], $p = .0076$) were verified in cervicovaginal samples from women positive for *C. trachomatis* infection.

DISCUSSION

Several studies have described that *C. trachomatis* clearance by the host involves innate and adaptive immune responses.[2,4] As part of this response, defensins are synthesized and released during the initial microbial infection. However, our data show lower levels of hBD-1, hBD-2, and hBD-3 in the cervical fluid of reproductive-aged women. So far, only 1 study has evaluated the cervicovaginal levels of β-defensins in relation to the status of C. *trachomatis* infection,[20] which demonstrated similar results

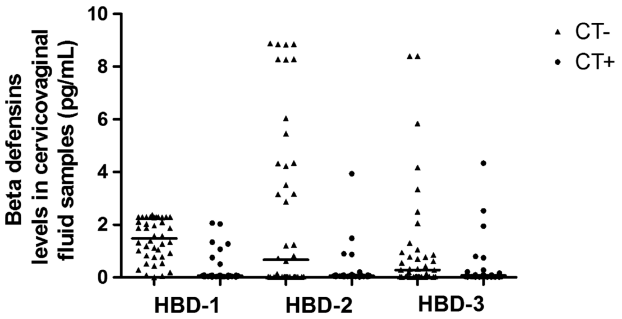

FIGURE 1. β-Defensin levels in cervicovaginal fluid samples from women negative (CT–) ($n = 37$) and positive for chlamydial endocervicitis (CT+) ($n = 37$). Horizontal bars represent median values. Mann-Whitney *U* test, $p < .05$.

to ours. Because β-defensin production stimulus is caused by the presence of pathogens and/or proinflammatory cytokines, we proposed the hypothesis that *C. trachomatis* has some mechanism of evading innate immunity that enables its persistence in the host.

Endocervical epithelial cells produce mucus, which forms a physical-chemical barrier to possible invading microorganisms. Besides mucin, mucus is composed of antimicrobial agents, including β-defensins. However, several bacteria have been detected, especially those related to bacterial vaginosis, which produce mucinases, sialidases, and proteinases as virulence factors to degrade mucus components, assisting their invasion.[21] There are no records on mucus and its constituents regarding hydrolytic enzyme production in *C. trachomatis*, but our results indicate strong evidence confirming this capability. This species produces more than 20 proteases essential for its development cycle, and their role in virulence is not fully elucidated.[22]

In disagreement with our results, Rizzo et al.[23] reported high levels of hBD-2 and interleukin 10 (IL-10) in human gingival fibroblasts after stimulation with *Chlamydia pneumoniae*. Similar results were obtained by Boldenow et al.,[24] who cultured amniotic epithelial cells, which expressed high concentrations of hBD-2, IL-1β, and tumor necrosis factor α in choriodecidua after stimulation by group B *Streptococcus.* Although Radtke et al.[25] observed hBD-1 constitutively expressed in a three-dimensional model of endocervical epithelial cells, they reported increased expression of this defensin and hBD-2 after exposure to microbial products.

Among the chlamydial proteases already described that participate in important host cells signaling pathways, the most relevant are tail-specific protease (Tsp) and chlamydial proteasome/protease-like activity factor (CPAF), which are activated when in contact with host cytoplasm.[26] Tsp can cleave NF-κB p65 in infected cells, blocking IL-1β, and consequently hBD-2 production.[26] There is evidence that CPAF has the same effect on NF-κB; however, this protease has several other substrates, such as transcription factors upstream stimulation factor (USF) 1 and regulatory factor X5, necessary for major histocompatibility complex (MHC) class I and II molecules expression. Therefore, CPAF inhibits MHC class II expression induced by IFN-γ, impairing infected cell recognition by T CD4+ lymphocytes. Moreover, this protease suppresses the constitutive and IFN-γ–induced expression of MHC class I, reducing T CD8+ lymphocyte recognition.[26] Thus, T CD8+ lymphocytes are not activated and do not produce IL-10, considered an important regulator of the initial inflammatory response to *C. trachomatis* infection, principally through the downregulation of proinflammatory cytokines.[27] Given the importance of immune response effector cells against *C. trachomatis*, inhibition of MHC hampers infection clearance.

Chlamydial proteasome/protease-like activity factor also ensures an antiapoptotic mechanism for *C. trachomatis*, enabling the maintenance of inclusion intracellular vacuole integrity, to limit the content exposure of the inclusion to cytoplasmic pathogen–associated molecular patterns, such as nucleotide-binding oligomerization domain-containing protein 1. This mechanism prevents the activation of pathways essential for the expression and production of cytokines and β-defensins by the host.[22] Furthermore, this protease ensures the evasion of caspase-1 pathway, involved in apoptosis activation and essential for IL-1β maturation.[22] Interleukin 1β, in turn, is important for inducing hBD-2, hBD-3, and hBD-4 production.[28,29]

Therefore, the lower levels of β-defensins detected could be due to the activation of proteases (CPAF, Tsp, and possibly others) that can alter intracellular signaling pathways and degrade the antimicrobial components present in endocervical mucus. This study clarifies new possibilities for research on hBD regulation during *C. trachomatis* infection by protease inhibitors.

CONCLUSIONS

Lower levels of hBD-1, hBD-2, and hBD-3 in cervicovaginal fluid were detected in the presence of C. *trachomatis* infection. Further studies must be conducted to clarify whether the proteolytic action of C. *trachomatis* on hBDs or the downregulation of production of these antimicrobial peptides is involved in this mechanism.

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