

RESEARCH ARTICLE

Cytological Features Associated with *Ureaplasma Urealyticum* in Pap Cervical Smear

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Abstract

Purpose: *Ureaplasma urealyticum* is associated with several obstetric complications and increases the importance of risk management in pregnant women. Furthermore, *U. urealyticum* has been identified as a cofactor that interacts with human papillomavirus infection in cervical cancer onset. The aim of this study was to assess specific cytological features of *U. urealyticum* infection in Pap smears to determine whether additional microbiological testing should be performed for pregnant women with a high possibility of *U. urealyticum* infection. **Methods:** Liquid-based cytology specimens (LBC) from cervical swabs of a total of 55 women, including 33 pregnant women who were negative for intraepithelial lesion or malignancy (NILM) on Pap testing and with *U. urealyticum* diagnosed without any other infectious microbes and 22 *U. urealyticum*-negative controls, were used in this study. We evaluated the localization of *U. urealyticum* by immunofluorescence, cytological features of secondary changes in squamous cells caused by inflammation, and the specimen background in Pap smears. **Results:** Based on analysis of Pap smears, a significant relationship was observed between *U. urealyticum* infection and cannonballs ($p < 0.05$) as well as predominance of coccoid bacteria ($p < 0.05$). A large number of *U. urealyticum* were detected in cannonballs by immunofluorescence. **Conclusion:** In the present study, cytological features in Pap smears of *U. urealyticum* infected samples, which have hardly been understood thus far, were assessed. The cytological features included cannonballs and predominance of coccoid bacteria. Our results might help in determining whether additional microbiological testing should be performed for pregnant women with a high possibility of *U. urealyticum* infection.

Keywords: *Ureaplasma urealyticum*- Pap test- cytological features- cannonballs- coccoid bacteria

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Introduction

Ureaplasma spp. is the smallest self-replicating micro-organism that can be grown in artificial medium. In Japan, *Ureaplasma urealyticum* is detected in as high as 40%–80% samples of vaginal secretion of sexually active women (Iwasaka et al., 1986). *U. urealyticum* is an indigenous bacterium present in the reproductive organs of adult women and thus, is mostly asymptomatic; however, it is associated with various obstetric complications, such as infertility, chorioamnionitis, stillbirth, and premature delivery, thereby increasing the importance of risk management in pregnant women (Abele-Horn et al., 1997, Waites et al., 2005, Volgmann et al., 2005).

In addition to obstetric complications, *U. urealyticum* has recently been shown to have a strong association with cervical intraepithelial neoplasm (CIN) and human papillomavirus (HPV) infection (Lukic et al., 2006, Biernat-Sudolska et al., 2011, Choi et al., 2014, Ji, 2017).

HPV is a sexually transmitted pathogen that is highly associated with cervical cancer; however, HPV infection alone does not lead to cancer onset (Verteramo et al., 2009). To date, it has been shown that cervical cancer onset is linked to smoking and contraceptive drug use, both of which act as environmental risk factors, and sexually transmitted pathogens, such as *Chlamydia trachomatis* (IARC, 2007). However, *U. urealyticum* has also drawn attention as a cofactor that interacts with HPV infection (Lukic et al., 2006, Biernat-Sudolska et al., 2011, Choi et al., 2014, Ji, 2017). Therefore, the importance of rapidly providing treatment to women infected with *U. urealyticum* has arisen (Lukic et al., 2006, Biernat-Sudolska et al., 2011). However, with regard to CIN onset and obstetric complications, the pathogenic factors of *U. urealyticum* remain controversial, and therefore, women diagnosed with *U. urealyticum* infection are generally not treated. Furthermore, Pap tests of *U. urealyticum*-positive patients have not been analyzed in

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detail, and thus, as expected, they have not been included in screening. Therefore, the present microbiological testing for *Ureaplasma spp.* is performed by clinicians as required on the basis of clinical symptoms of patients.

The aim of the present study was to assess specific cytological features of *U. urealyticum* in Pap smears to determine whether additional microbiological testing should be performed for pregnant women with a high possibility of *U. urealyticum* infection.

Materials and Methods

Clinical samples

Among all pregnant women who were examined at the outpatient services of Fukui Maternity Clinic from 2009 to 2014, cervical swabs were collected from patients who underwent routine Pap smear screening while at the same time underwent microbiological testing for cervical infection. The cases were selected according to the Pap smear test based on the 2001 Bethesda System as well as according to a database search of screening test results for *Neisseria gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, *Candida albicans*, herpes simplex virus (HSV), HPV, *Mycoplasma genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum*. *N. gonorrhoeae* and *C. trachomatis* were tested using the strand displacement amplification (SDA) assay; *Mycoplasma spp.* and *Ureaplasma spp.* were tested using the PCR-Invader® assay; and the HPV was tested using a PCR assay with specific primers for high-risk type. *T. vaginalis*, *C. albicans*, and HSV were morphologically diagnosed according to the Pap test.

In the present study, only patients negative for intraepithelial lesion or malignancy (NILM) on Pap testing and with *U. urealyticum* diagnosed without any other infectious microbes identified by microbiological test were selected. Samples that were negative for all of the aforementioned infectious microbes were included as the negative control. A total of 55 patients met these criteria, including 33 patients positive for *U. urealyticum* and 22 negative controls, and liquid-based cytology (LBC) specimens from the cervical swabs of these patients were used in the study. The mean age of patients was 28.5 years (range, 20-36 years) for *U. urealyticum*-positive; 29.7 years (range, 20-38 years) for the negative control. This study was approved by the ethics committee of Kyorin University Faculty of Health Sciences.

U. urealyticum detection by immunofluorescence

LBC specimens were centrifuged at 1,500 rpm for 5 min. The cell pellet was spread between two glass slides by the pull-apart method. Once completely dry, the slides were placed in 95% ethanol. The slides were treated with a protein block serum-free (Dako, Tokyo, Japan) for 10 min for inactivation, followed by addition of polyclonal antibody against *U. urealyticum* (ab24357; Abcam, Tokyo, Japan) at a dilution of 1:50 in 0.01 M phosphate-buffered saline (PBS), pH 7.4 for 1 h at room temperature. Next, the slides were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-chicken IgY H and L (ab46969; Abcam, Tokyo, Japan) at a dilution of 1:100 in PBS for 1 h at room temperature. The slides were washed

and counterstained using 4', 6'-diamidino-2'-phenylindole dihydrochloride (236276; Roche Diagnostics, Mannheim, Germany). Localization of *U. urealyticum* was observed with a confocal laser scanning microscope.

Morphological evaluation of *U. urealyticum* infected specimens

Using the cell pellet of each LBC specimen, samples were prepared using the two slide method, followed by Papanicolaou staining. In *U. urealyticum*-positive and *U. urealyticum*-negative Pap smears of cervical swabs, we focused on secondary changes in squamous cells caused by inflammation and the cytological features of the specimen background. The cytological features included nuclear enlargement of squamous cells, reactive nuclear changes in chromatin hyperstaining, perinuclear halo, eosinophilic staining of cell cytoplasm, cannonballs characterized by compact clusters of neutrophils on squamous cells, predominance of coccoid bacteria, clue cells, and neutrophil count. Cells were considered to be positive for cannonballs when 30 or more compact clusters of neutrophils appeared on the squamous cells per specimen. Clue cells were considered positive when the number of squamous cells with abundant bacteria attached accounted for 20% or more of overall squamous cells, on the basis of the diagnostic criteria of bacterial vaginosis by Amsel (1983). Neutrophilia was considered if the total number of neutrophils observed in the five high-power field (40× objective) was 1,000 or more.

Statistical analysis was performed using SPSS version 21.0 (SPSS, Chicago, IL). Differences between groups were examined using the χ^2 Fisher's exact probability test or the Mann-Whitney U test according to the characteristics of data distribution. P-values < 0.05 were considered to be statistically significant.

Results

The frequency of appearance of each cytological feature observed in each Pap smear of a cervical swab sample infected with *U. urealyticum* are shown in Table 1. The cytological feature with the highest frequency in *U. urealyticum*-infected samples was cannonballs

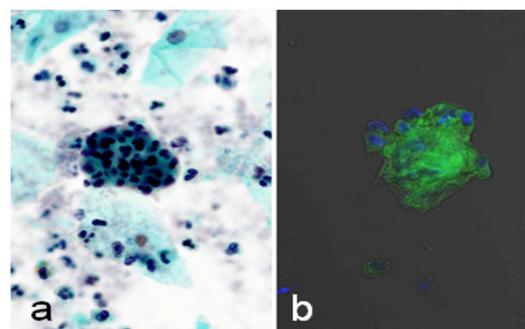


Figure 1. Cannonballs and Coccoid Bacteria are Cytological Features Present in *Ureaplasma Urealyticum* infected Cells. a) Cannonballs characterized by compact clusters of neutrophils were observed at the center of the photograph. Coccoid bacteria were observed around the cannonballs. (Pap. staining 40x). b) Immunofluorescence images of *U. urealyticum* in cannonballs (40x).

Table 1. Cytological Findings of *Ureaplasma Urealyticum* in Pap Cervical Smears

Cytological findings	<i>Ureaplasma urealyticum</i>		p-value
	Positive N = 33	Negative N = 22	
Reactive nuclear change +	12	10	0.3461
Perinuclear halos +	7	7	0.2828
Eosinophilic staining +	15	11	0.4776
Cannonballs +	22	8	0.0262
Predominance of coccoid bacteria +	17	4	0.0123
Clue cells +	13	12	0.2689
Large number of neutrophil +	5	2	0.4113

(Figure 1a), followed by a predominance of coccoid bacteria. Chi-square test showed a significant correlation only for cannonballs ($p < 0.05$) and predominance of coccoid bacteria ($p < 0.05$). Other than these, no significant relationship was observed between other cytological features and *U. urealyticum* infection. In smears of *U. urealyticum*-positive cervical swabs, *U. urealyticum* was detected by immunofluorescence in almost all squamous cells and neutrophils on the specimens. Cannonballs exhibited a strong fluorescent signal and had a large amount of *U. urealyticum* (Figure 1b).

Discussion

In *U. urealyticum*-positive smears of cervical swabs, *U. urealyticum* was detected on the surface of squamous cells and neutrophils. It has been shown previously that *Ureaplasma spp.* attach to various human cells, including urethral epithelial cells (Shepard et al., 1979), neutrophils (Viscardi et al., 2009), and *Mycoplasma spp.*, which is considered important because even if the host cell surface localization and adhesion are not transferred inside the cell, it has the ability to continue to form pathological lesions after colony formation (Waites et al., 2005). The proposed pathogenic factors by which *U. urealyticum* directly damages cells (Crouse et al., 1990, Li et al., 2000, Glass et al., 2000) include oxygen free radicals formed by hyperoxia, ammonia, and hydrogen peroxide, while immunological indirect factors include cytokines (Manimtim et al., 2001, Jones et al., 2010). It was inferred that these pathogenic factors did not cause morphological changes that could be observed on an optical microscope. Previously, specific cytological features were not found in *U. urealyticum*-infected Pap smears (Lee et al., 1999); therefore, perhaps the specimen background should be focused on in addition to epithelial cells.

The results of the present study demonstrated that a feature presumed to indicate *U. urealyticum* infection in Pap smears of cervical swabs was a large number of cannonballs located in the specimen background. This finding is known to often appear in Pap smears during *T. vaginalis* infection (Hwang et al., 2011). It has been reported that during *Trichomonas* infection, there are elevated levels of interleukin-8 (IL-8), a cytokine that induces neutrophil migration (Kalinka et al., 2006),

and it is assumed that cannonballs appear in this way. Similarly, the stimulatory effect of *Ureaplasma spp.* on cytokine release has been confirmed in vitro. It has been reported that in early monocytes from cultured human umbilical blood, *U. urealyticum* stimulates TNF- α and IL-8 production and if administered simultaneously with gram-negative lipopolysaccharides, the formation of pre-inflammatory cytokines may be markedly increased (Jones et al., 2010, Manimtim et al., 2001). While cannonballs also appear in Pap smears without *Trichomonas* infection, it might be one cytological feature suggestive of *U. urealyticum* infection. It has previously been shown that *U. urealyticum* causes responsive cell changes (Choi et al., 2014); however, the details were unclear. In Pap smears of *U. urealyticum* infection, the fact that a large number of cannonballs appear despite there being no increase in predominance of neutrophil in the specimen might be a new finding that differs from *Trichomonas* infection.

We also found that a large number of coccoid bacteria were present in *U. urealyticum*-infected Pap smears. The presence of coccoid bacteria can be explained by the increased pH level due to ammonia production as a result of *Ureaplasma spp.* with urease activity (Grenabo et al., 1988, Smith et al., 1993). Mohon (1986) reported that in vaginal *U. urealyticum* infection, the prevalence of bacterial vaginosis can increase by approximately two-fold and the density of bacterial flora other than *lactobacillus* can increase by 100-fold. Therefore, it was suggested that, *U. urealyticum* infection might have a causal relationship with the onset of bacterial vaginosis as a result of abundant coccoid bacterial growth, as was indicated in these studies. Krishnamurthy (2016) also demonstrated a significant relationship between cannonballs and bacterial vaginosis, which is consistent with our results. Although they did not investigate the relationship with *U. urealyticum* infection in their study, it is possible that the presence of *U. urealyticum* affected the relationships between cannonballs and bacterial vaginosis-derived coccoid bacteria. It should be noted that there were few samples analyzed and there were residual confounding variables caused by unknown factors that could not be eliminated, both of which confer limitations to the present study. To demonstrate the significance and clinical importance of these cytological features, further studies are needed with a larger sample size. In particular, comparisons need to be made between Pap smear samples from patients with *U. urealyticum* infection with and without the presence of other sexually transmitted pathogens, including HPV, and the question of whether detection rates of cannonball and coccoid bacteria are affected by other infectious factors needs to be investigated.

In conclusion, the present study showed that cannonballs and coccoid bacteria were cytological features in Pap smears of *U. urealyticum* infection, whose significance was previously unclear. Our results might help in determining whether pregnant women at high risk of *U. urealyticum* infection should undergo additional microbiological testing.

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Conflict of interest disclosures

The authors made no disclosures.

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