Association of Chlamydia trachomatis, Mycoplasma spp., Ureaplasma urealyticum and U. parvum with Human Papillomavirus in Patients with Cervical Cancer

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ABSTRACT
Besides human papillomavirus (HPV), the cervical carcinogenesis is also affected by many risk factors including pathogenic bacteria such as Chlamydia trachomatis (CT), Mycoplasma spp. (MS), Ureaplasma urealyticum (UU), and U. parvum (UP) infections. Thus, we studied the bacterial infections for 68 patients with cervical cancer cases and other cervical problems. Cervical swab samples were collected by specialist doctors and tested for the pathogenic bacteria by real time polymerase chain reaction (rPCR) and HPV by conventional PCR. Of 68 patients, 22 were diagnosed as cervical cancer and 46 were diagnosed as other cervical problems. All patients with cervical cancer were HPV positive, while the patients with other cervical problems were HPV negative. Of 22 HPV positive-cervical cancer patients, 7 (31.82%), 6 (27.27%), 3 (13.64%) were positive for MS, UU, and UP, respectively. None of the patients was CT positive. For 46 HPV negative-other cervical problem patients, 6 (13.04%), 12 (26.09%), 20 (43.48%), and 22 (47.83%) were positive for CT, MS, UU, and UP, respectively. There was no association of CT, MS, and UU infections with the HPV positive-cervical cancer patients (p >0.05). However, there is a negative correlation between UP infection and the HPV negative-other cervical problem patients (p<0.05).

Keywords: HPV, Cervical cancer, Chlamydia, Mycoplasma, Ureaplasma

1. INTRODUCTION
Cervical cancer is ranked 4th of several types of cancer that most attack women in the world. Persistent infections HPV have been reported to be the primary aetiology of cervical cancer [1]. The incidence of cervical cancer in Indonesia ranks second with a total of 20,928 cases/year and a mortality rate of 9,498 people/year [2].

Based on these data the mortality rate caused by cervical cancer is quite high because it almost reaches 50% of the morbidity/year. Human papillomavirus (HPV) has been identified as an etiological agent of cervical cancer warts. Among sexually transmitted infections (STIs), only HPV infections are known to be the main cause of cervical cancer [3]. HPV was detected in 90% of cervical cancer cases, with the majority of HPV18 and HPV16 subtypes [1] [4]. However, cervical carcinogenesis is not facilitated by HPV infection alone, but also related to environmental factors, such as many sex partners and sexually transmitted diseases [5].

At present from various STI agents, several studies have reported that infections caused by C. trachomatis, Mycoplasma spp., U. urealyticum, U. parvum play an important role in the persistence of HPV infection, causing cervical cancer [6-8]. According to the World Health Organization [9], more than one million sexually transmitted infections (STIs) are obtained every day. C. trachomatis infection is known to inhibit apoptosis of infected cells, [6, 10] while Mycoplasma spp. infection can suppress cell-mediated immunity [11, 12]. This mechanism makes infected cervical cells more susceptible to HPV infection and causes persistence of HPV [11]. Research by Adebamowo et al. in Nigeria states that there is a significant relationship between
Mycoplasma spp. persistent between the vaginal microbiota and persistent hr-HPV infection [12]. Jensen at al. study in Denmark reported that women with positive hrHPV with recurrent C. trachomatis infection increased the risk of CIN 3+ [13]. Research by Lukic et al. in Italy showed that the interaction between HPV and Ureaplasma in concomitant infections had an important role in the development of lesions precancerous cervix or cancer [5]. The chance of HPV persistence is also caused by Ureaplasma infection with increased expression of E6 HPV oncogenes in cervical cells and can cause abnormal cervical cytology [14].

To our knowledge, there have been no reports from Indonesia regarding the association of infection C. trachomatis, Mycoplasma spp., U. urealyticum, and U. parvum as risk factors for HPV infection in cervical cancer women. Therefore, we conduct research with this cross-sectional design to determine the relationship of these infections as a risk factor for HPV infection in women with cervical cancer in Indonesia.

2. MATERIAL AND METHODS

2.1. Clinical Specimen

The cervical swab samples used in the present study were stored samples collected from five clinics in Makassar, South Sulawesi, Indonesia between July 2014 and November 2014. All samples had undergone PA (anatomical pathology) for cervical cancer cases and Pap smear examination to determine noncervical subjects. Related the sampel were stored from -80 °C, the exclusion criteria were: 1) Negative β- Globin test; 2) sample size under 400 µL. Sample size are 68 consist of samples cervical cancer, pelvic inflammatory disease (PID), flour albus, Chronic inflammation, and normal cervix. The study was stated to have passed the ethical studies with letter number 0247/UN2.F1/ETIK/2018 from the Ethics Committee of the Medical Research of the University of Indonesia.

2.2. DNA extraction for bacteria identification

The swab specimens used were stored in sterile phosphate-buffered saline on a 1.5-mL microcentrifuge tube at −80°C. The extraction of cervical swab specimen DNA was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Jerman). The procedure used was incorporated to the established manufacturing protocols with a final solution volume of 60 µL that was stored at −20°C until being used.

2.3. SYBR Green real time PCR for bacteria identification

Before samples included in this study, the sample collection was validated by PCR human β-Globin with modified primers (Forward: 5′ ‘AAG AGC CAA GAA GGT A 3′ and Reverse: 5′ ‘AAC TTCATC CAC GTT CAC 3′) [15]. For HPV, the virus was detected by using consensus primers (GP5+: 5′-TTT GTT ACT GTG GTA GAT ACT AC-3′ and GP6+: 5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′) as reported previously by de Roda Husman et al [39].

For bacteria, the primers used in this study are the primers that have been used in previous studies that have been reported by Dhawan et al for [16], Pascual et al [17] Jensen et al [13]. C. trachomatis with a product size of 71 bp (Ctr forward 5′-CATGAAAACTCGTTCCGAAATAGAA-3′ and Ctr reverse 5′-TCAGAGCTTTACCTAAACACCGTATA-3′) (16); Mycoplasma spp. with a product size of 101 bp (Mh forward 5′-TGGTGCAAGTCTGCAACGATA-3′ and Mh reverse 5′-CCCCACCTCTCCCGAGTTA-3′) (17); U. urealyticum with a product size 127 bp (forward: 5′-GCA AGA AGA CGT TTA GCT AGA GGT TT-3′ dan reverse: 5′-CAC GAG CATT GTA TTA AGT CAG-3′); and U. parvum with a product size 99 bp (forward: 5′-GAT CAC ATT TTC ACC TTG TTT GAA GTG-3′ dan reverse: 5′-AAC GTC GTC CAT AAG CAC TTT G-3′) [13].

The PCR reactions were performed by the following compositions (20 µL): 1x qPCR Bios SYGreen Mix (PCR Biosystems, London, UK); 0.5 µM (β-globin, U. urealyticum, U. parvum), 0.2 µM (Mycoplasma spp.), or 0.8 µM (C. trachomatis) of forward primers; 0.5 µM (β-globin, U. urealyticum, U. parvum), 0.2 µM (Mycoplasma spp.), or 0.4 µM (C. trachomatis) of reverse primers; and 4 µL of DNA template. PCR thermal cycling (LightCycler 2.0; Roche Holding AG, Basel, Switzerland) was performed with the following conditions: 95°C for 5 min and then 45 cycles of 95°C for 10 sec and 64°C (U. urealyticum) or 60°C (β-globin, U. parvum, Mycoplasma spp) or 56°C (C. trachomatis) for 30 sec. The PCR results were analyzed by melting curve patterns. Positive PCR results were confirmed by DNA sequencing.

2.4. Data analysis

The data that were obtained were analyzed using a Chi-squared statistical test and Fisher test with p value of 0.05 was considered to be statistically significant.

3. RESULT AND DISCUSSION

3.1. Subject characteristics

The subjects were further divided into the two groups of employees (47.5%) and housewives (52.9%). The average age subject with the cervical cancer were 46.5 years and the average subject with other cervical
problem were 38.6 years. This subject consist of cervical cancer, PID, flour albus, Chronic inflammation, and normal cervic with percentage, 32.35%, 10.29%, 8.82%, 32.35% respectively.

### 3.2 SYBR Green real-time PCR result

For bacterial detections, real-time PCR (rPCR) results showed several graphical patterns, except for *C. trachomatis* (Figure 1A). The *Mycoplasma* spp. rPCR results revealed three melting temperature (Tm) peaks (Figure 1B). Of the three peaks, a peak with a Tm of 80°C was positive, while the other two were negative (i.e., Tms of 84°C and 86°C, respectively). For *U. urealyticum*, rPCR showed three Tm peaks, with two peaks being positive at 79 °C and 80°C and one peak being negative in accordance with the negative control (Figure 1C). For *U. parvum*, rPCR showed two Tm peaks, with one being positive (76°C) and one being negative (74°C) (Figure 1D). All samples that showed different patterns were confirmed by DNA sequencing (data not shown).

The real time PCR result (Data not shown) revealed all of Sample collection and DNA extraction were validated by PCR conducted using standard β-globin (Housekeeping gene). Of 68 patients, 22 were diagnosed as cervical cancer, while 46 were diagnosed as other cervical problems. All patients with cervical cancer were HPV positive, while the patients with other cervical problems were HPV negative. For *C. trachomatis* detection in the patient with cervical cancer status there were no positive samples, while in the patients with other cervical problems there were 6 positive samples from a total of 46 samples. Fisher test results for *C. trachomatis* bacteria p value of 0.086. In the *Mycoplasma* spp. detection in the patient with cervical cancer status there were 7 positive total samples from 22 samples, while the patients with other cervical problems there were 12 positive samples from a total of 46 samples. Based on Chi-square test obtained p value 0.622. Furthermore, the *U. urealyticum* test in patient with the cervical cancer status there were 6 positive total samples from 22 samples, while the patients with other cervical problems there were 20 positive samples from a total of 46 samples. Using Chi square test obtained p value 0.198. Then the parvum test in patient with the cervical cancer status there were 3 positive samples from a total of 22 samples, while the patients with other cervical problems there were 22 positive samples from a total of 46 samples. Using the Chi square test, the p value of 0.006 shows that the association between infection for HPV infection has a correlation other cervical problems (Table 1).

### 3.3. Discussion

*C. trachomatis* has been reported to be the most etiological a gent causing STIs across the world, with approximately 89 million cases/year [6]. These infections may lead to persistent infections and inflammation, leading to the inhibition of cell apoptosis and metaplasia in cervical squamous cells, thereby cause the cells susceptible to HPV infection [6,18]. Jianhua et al’s research in China reported that the presence of *C. trachomatis* was associated with HPV infection [20]. Laura et al. study in Paraguay and Nadja et al. in Kenya also reported the presence of *C. trachomatis* infection in HPV positive patients [21, 25]. The results of the present study showed no significant association between *C. trachomatis* with HPV infection in the patient with cervical cancer status (Table 1). Previous research with findings that are in line with the results of this study include the studies by Andre et al. and Mayara et al. in Brazil and Panatto et al. in Italy, respectively [19,23-24]. Panatto et al. reports that there is no significant relationship between *C. trachomatis*
There is controversy regarding C. trachomatis prevalence varied geographically [19]. Second, regarding the study population, several studies used subjects with different age ranges; Panatto et al. for example did not find a significant relationship between C. trachomatis and HPV in several age groups (i.e., those aged 21–23 years and 24–26 years, but did find such in one age group of individuals aged 18–20 years) [19].

In addition to C. trachomatis, U. urealyticum and U. parvum tests were also conducted in the present study. There is controversy regarding U. urealyticum as a pathogen in STIs. However, a recent meta-analysis study suggested that U. urealyticum was the etiology of agents in STIs [26]. A recent study showed that the presence of high Ureaplasma levels can be attributed to STIs [21]. In this study, we found we found no significant association infection U. urealyticum and U. parvum with HPV infection. This result is consistent with the findings of Laura et al. research in Parguay, Yamakazi et al. in Japan and Kim et al. in Korea [21, 27, 28]. However, studies by Camporiando et al. in Italy and Biernat et al in Poland indicated that there were significant relationships between U. urealyticum and U. parvum infection and HPV infection [8, 29]. However, U. parvum infection showed a significant association with the HPV negative-other cervical problem status and normal cervical. Based on several studies, adequate evidence has not been reported that M. hominis, U. parvum or U. urealyticum infections in women cause inflammation of vulvovaginitis, cervicitis, urethritis, PID or infertility [20,31–34]. However Women of reproductive age often suffer from urogenital infections. The prevalence of UP in a cohort of healthy nonpregnant women is 57%, which is a much higher prevalence compared to other genital mycoplasmas, viruses, Chlamydia or GBS (beta-haemolytic Group B Streptococcus), infection [35].

The bacterium can be found as a commensal agent in healthy women, but it can be a harmful agent in sexually active women [11, 36]. Several studies have reported the association between M. hominis infection and cervical problems [36]. A study reported that women with M. hominis infection could suppress the cell-mediated immunity, an important protective mechanism against HPV infection [11, 12]. The detection results of Mycoplasma spp. showed that there was no found significant association and HPV infection in the patient with cervical cancer status, but the odds ratio was 1.3, which means that the ratio of Mycoplasma spp. infection in the HPV positive sample were 1.3 times greater than that in the in the patients with other cervical problems.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Cervical cancer n (%)</th>
<th>Other Cervical Problems n (%)</th>
<th>Total n (%)</th>
<th>p-value</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0 (0)</td>
<td>6 (13.04)</td>
<td>6 (8.82)</td>
<td>0.086a</td>
<td>NA</td>
</tr>
<tr>
<td>Negative</td>
<td>22 (100)</td>
<td>40 (86.96)</td>
<td>62 (91.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycoplasma spp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7 (31.82)</td>
<td>12 (26.09)</td>
<td>19 (27.94)</td>
<td>0.622b</td>
<td>1.3</td>
</tr>
<tr>
<td>Negative</td>
<td>15 (68.18)</td>
<td>34 (73.91)</td>
<td>49 (72.06)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U. urealyticum</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Positive</td>
<td>6 (27.27)</td>
<td>20 (43.48)</td>
<td>26 (38.24)</td>
<td>0.198b</td>
<td>0.4</td>
</tr>
<tr>
<td>Negative</td>
<td>16 (72.73)</td>
<td>26 (56.52)</td>
<td>42 (61.76)</td>
<td></td>
<td></td>
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<tr>
<td>U. parvum</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Positive</td>
<td>3 (13.64)</td>
<td>22 (47.83)</td>
<td>25 (36.76)</td>
<td>0.006b</td>
<td>0.1</td>
</tr>
<tr>
<td>Negative</td>
<td>19 (86.36)</td>
<td>34 (73.91)</td>
<td>43 (63.24)</td>
<td></td>
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</tr>
</tbody>
</table>

OR, Odds ratio
a Fisher test
b Chi square test

DNA prevalence and positive HPV status based on several age groups [19]. Inconsistency of study results related to C. trachomatis and HPV infections can be caused by several factors. First, the differences in research locations reported that C. trachomatis prevalence varied geographically [19]. Second, regarding the study population, several studies used subjects with different age ranges; Panatto et al. for example did not find a significant relationship between C. trachomatis and HPV in several age groups (i.e., those aged 21–23 years and 24–26 years, but did find such in one age group of individuals aged 18–20 years) [19].
In line with these findings, Kim et al. in Korea, Adebamwo et al. in Nigeria, and de Abderu et al. in Brazil also found no significant association between Mycoplasma spp. infection (Mycoplasma hominis and Mycoplasma genitalium) and HPV infection [12, 28, 37]. However, in contrast to the present study, Biernat-Sudolska et al. and Ekiel et al. in Poland reported in their studies that there was a significant association between Mycoplasma spp. and HPV infection [29, 38].

4. CONCLUSION

There is no association of the four pathogenic bacteria with the HPV positive-cervical cancer status. The UP infection showed a significant association with the HPV negative-other cervical problem status.

REFERENCES


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