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Detection of Candida in Cervical Cancer Tissue by Periodic Acid Shiff and Gomori Methenamine Silver Staining

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KEYWORDS

Cervical Cancer, Candida, Periodic Acid Schiff, Gomori Methenamine Silver, Different staining methods

ABSTRACT:

Introduction and Aim: Cervical cancer is a global health burden. Of all the cancer incidences reported in Indonesia, cervical cancer occupies the second position as the highest contributor to the incidence throughout the year, and the third position is the leading cause of death. Tissue culture and staining methods were used to diagnose the cervical cancer. This study aimed to compare Candida detection and the possibility of vulvovaginal candidiasis risk factors in cervical cancer tissue blocks stained with periodic acid-Schiff (PAS) and Gomori methenamine silver (GMS).

Methods: This was an analytical observational study using a cross-sectional approach. This study compared PAS and GMS staining to detect Candida fungi in paraffin block tissue from individuals diagnosed with cervical cancer at the Anatomical Pathology Laboratory of Kediri Regency Regional General Hospital in 2021–2022. The sampling used Total sampling was carried out from March to December 2022. Data analyses were descriptively performed.

Results: The incidence of cervical cancer was dominated by patients aged 51–60 years who belonged the decade 6. In this study, 32 blocks of cervical cancer tissues were deparaffinized and analyzed. A histopathological diagnosis of the examined cervical biopsy samples was obtained. A total of 81.25% had cervical squamous cell carcinoma, in 18.75% of the patients and cervical adenocarcinoma. The findings of this study show that staining with GMS can detect more Candida fungi than PAS staining. Microscopic examination showed that GMS staining had a more obvious contrast, marked by black structures in the green area. Thirteen samples (40.7%) of the 32 examined for vulvovaginitis. However, this study did not show that Candida invasion of the cervix is a risk factor for cervical cancer.

Conclusion: PAS and GMS staining can be used to detect Candida fungi in cervical cancer tissue. GMS staining has higher accuracy in detecting Candida in cervical squamous cell carcinoma and cervical adenocarcinoma. The risk factors for fungal infections and the occurrence of cervical cancer require further investigation.

INTRODUCTION

Cervical cancer has become a global and national health burden in Indonesia. Cervical cancer is characterized by abnormal changes in the ability of cells to form tissues, resulting in abnormal bleeding, pelvic pain, abnormal vaginal discharge, and other manifestations [1]. The incidence of cervical cancer has significantly increased over the last two decades. According to a World Health Organization (WHO) report, in 2021, there were 36,633 new cases (9.2%) of cervical cancer in Indonesia with a cumulative risk of 2.69, while reported death cases reached 21,003 (9%) with a cumulative risk of 1.73. In the last five years, 92,930 new cases have been reported

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across all age groups, with a prevalence of 68.43 per 100,000 people [2, 3]. Cervical cancer is the third-highest cause of death and contributes to the second-highest number of cases, as well as being one of Indonesia's largest health financing burdens [4].

Human papillomavirus (HPV) infection is the main cause of this disease in approximately 95% of cases [5]. Various risk factors have been shown to increase the incidence of cervical cancer. Predispositions that are often reported include sociodemographic factors, including age, occupation, socioeconomic status, and sexual activity factors, including age at first sex, exposure to diethylstilbestrol (DES), sex with many sex partners, parity, low genital hygiene, smoking, history of venereal disease, chronic trauma to the cervix, not doing the HPV vaccine, and oral contraceptive use (larger than four years) [6, 7]. This has certainly contributed to an increase in the number of cervical cancer cases. However, in many cases, this condition is not realized in the early phase and is diagnosed when entering the early or advanced stages [7, 8].

The microbiota has been extensively studied with respect to the incidence of cervical cancer. The Exo/ectocervix region becomes a breeding ground or habitat for normal flora in the vagina [9]. Several studies have shown that certain human papillomavirus (HPV) serotypes are believed to cause cervical cancer. In cervical samples with high-risk HPV and introitus samples from people with atypical squamous cells of undetermined significance (ASCUS), a much larger variety of fungi was found. Sporidiobolaceae and Saccharomyces for ASCUS, and Malassezia for high-risk HPV infection are fungal biomarker markers for the vagina and cervix. According to these findings, certain bacterial and fungal communities in the cervical squamous epithelium may play a role in cervical dysplasia [10].

Research conducted at the Outpatient Unit of Skin and Genital Health at Dr. Soetomo Hospital Surabaya in 2012–2014 found that most patients were married (72.9 %) and only 24.6% were unmarried. When a person is married, sexual activity increases. Sexual intercourse can increase pH in the vaginal area. Lactobacilli are normal flora that are important for providing resistance to prevent the invasion or development of Candida [11, 12]. There are numerous pathogenic species of the genus Candida. *Candidiasis* is an infection caused by all the Candida species. *Candida albicans* is a naturally occurring fungus. It is present in 40–80% of healthy individuals. Candida is a commensal or pathogenic organism found in the mouth, intestine, and vagina. Candida infections typically result from alterations in cellular immunity, normal flora, or normal physiology [13].

Diagnosis of invasive or chronic fungal infections is challenging, especially in immunocompromised individuals [14]. Chronic mucocutaneous candidiasis is characterized by frequent or chronic *Candida albicans* infections of the skin, nails, and oral and vaginal mucosa [15]. Several methods have been used to diagnose diseases caused by fungi, including tissue culture and staining. However, several fungi can only be detected using tissue staining [16, 17]. The high risk of invasiveness from Candida, especially in individuals infected with cervical cancer, makes the development of the best detection method with high accuracy necessary to increase the speed of diagnosis while identifying the early occurrence of cervical cancer, including the presence of invasive fungi [18].

To date, there have been limited studies comparing the staining of cervical cancer tissue and the detection of Candida fungi using periodic acid-Schiff (PAS) staining and Gomori methenamine silver staining (GMS). This is important to fill in the gaps in the literature and provide up-to-date information regarding the accuracy, diagnostic methods, and identification of invasive pathogens in the tissues of individuals with cervical cancer. This study aimed to compare Candida detection and the possibility of vulvovaginal candidiasis risk factors in cervical cancer tissue blocks using periodic acid-Schiff (PAS) staining and Gomori methenamine silver (GMS). Hopefully, this study will provide new information related to fungal staining that can better detect Candida in tissue pieces diagnosed with cervical cancer and can be applied as a permanent procedure to detect Candida in tissue pieces with a cervical cancer diagnosis in public services.

MATERIALS AND METHODS

Study Design

This study used an analytical observational research design with a cross-sectional approach [19]. Paraffin-embedded tissue samples were obtained from patients diagnosed with cervical cancer at the Anatomical Pathology Laboratory of the Kediri Regency General Hospital between 2021and 2022. Total sampling was conducted over one period (years) [20]. The study was conducted from March 2022 to December 2022.

Tools and Materials

In this study, the tools and materials used included aqua dest, chromic acid (5 %), sodium bisulfite (1 %), gold chloride (0.1 %), sodium thiosulfate (2 %), and light-green aqua dest (GMS). In contrast, Schiff, ammonia

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water, gill's hematoxylin, 1% periodic acid solution, timers, and preparation sets were used for periodic acid Schiff (PAS) staining, as well as microtomes and Nikon light microscopes to support research.

Research Procedure

Cutting of preparations

At this stage, the tissue embedded in the paraffin block was adjusted to fit on the microtome and then cut thinly to a thickness of 3-5 m, resulting in a representative paraffin tape cut to facilitate sample identification. The stage of cutting the preparation begins by installing a microtome knife, setting the thickness of the cut between 3-5 m, preparing a floating bath and putting water into it, and then setting the temperature between 25 °C and 30 °C. Then, take the network block, and mount it on the block hook on the microtome, the block hook lock. If properly installed, prepare a glass object, smear it with glue, and code it. The microtome was unlocked and the surface of the block was flattened until a complete picture of the network was observed. Furthermore, microtome locks cut the tissue by turning the microtome lever quickly and regularly, so that the cut results were paraffin tape representing the block. The paraffin tape was then stretched and transferred to a floating bath. The paraffin tape was captured with a glass object smeared with glue. The samples were dried in an incubator at 60–65 °C for 20 min in the final stage.

Gomori Methenamine Silver (GMS) staining

Gomori Methenamine Silver (GMS) staining is performed to identify Candida in pieces of tissue with a cervical cancer diagnosis. GMS staining is the gold standard for diagnosis. GMS staining begins with preparing preparations that have been deparaffinized, adding 5% chromic acid to cover the entire tissue, and incubating for 1 h. After rinsing with an aqueous solution until clean, this was done 3–4 times. The next step was to prepare a working solution of methenamine silver in a water bath at a temperature of 58 °C for approximately 10 min. The preparation was then placed in a water bath and identified under a microscope with the criterion that the tissue must be yellowish-brown. If expected, paint with 0.1% gold chloride using a Pasteur pipette until it covers the tissue; wait up to 5 min, rinse, and continue administering sodium thiosulfate 2%. The preparation was then dripped with light green and allowed to stand for up to 30 s if it had been rinsed with 70% alcohol, placed in a glass object, and covered with a glass cover. Finally, entellan was added and the samples were observed under a microscope.

Periodic Acid Schiff (PAS) staining

At this stage, deparaffinized preparations were taken and dripped with 1% phenolic acid using a Pasteur pipette. After rinsing for 15 min with Aquadest, clean water was added for 5 min to rinse the preparation. The preparation was then dripped with Schiff reagent, allowed to stand for 15 min, and rinsed with aquadest. Finally, the sections were stained with Gill's hematoxylin, allowed to stand for 3 min, and rinsed. The preparation was dipped in ammonia water until bluish-red, dehydrated, and observed under a microscope.

Ethical Approval

This research was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga number 196/EC/KEPK/FKUA/2022, and a research permit was obtained at the Kediri Regency Regional General Hospital by the Kediri Regency National and Political Unity Agency (HM.04.4_1518/418.62/XI/2022.

Data analysis

The data collected were then tabulated and analyzed descriptively, and are presented as frequency positive values and percentages accompanied by images of the identification of Candida in the study sample.

RESULTS

The study results show that the frequency of cervical cancer patients who have undergone histopathological examinations in the period 2021–2022 at Kediri Regency Hospital reached 32 paraffin blocks. Patients between the ages of 51 and 60 years comprise most of the population based on this value. This indicates that patients with cervical cancer are older. Paraffin block examinations of patients with cervical cancer, based on age, are presented in Table 1.

Table 1: Paraffin block examination of cervical cancer patients based on age

Age of Respondents	Frequency	Percentage (%)
Decade 4 (30 – 40 years)	3	9.5
Decade 5 (41 – 50 years)	8	25
Decade 6 (51 – 60 years)	12	37.5
Decade 7 (61 – 70 years)	9	28
Total	32	100

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Based on the histopathological examination of cervical cancer tissue, patients obtained the type of cervical squamous cell carcinoma dominated by Gomori methenamine silver (GMS) in 10 out of 26 paraffin blocks. In contrast, the cervical adenocarcinoma type was confirmed to be positive based on GMS staining. These results indicate that staining using the periodic acid-Schiff (PAS) method is less than that using GMS. The coloring sensitivity of GMS was better than that of PAS. Two of six cervical adenocarcinomas were confirmed by GMS staining. The results of the cervical cancer tissue examination using PAS and GMS are presented in Table 2 and Figure 1.

Table 2: Staining of cervical cancer tissue using PAS and GMS

Examination	Frequency	Percentage (%)	Coloring (positive)	
			PAS	GMS
Squamous Cell Carcinoma of the cervix	26	81.25	8	10
Cervical adenocarcinoma	6	18.75	1	2
Total	32	100	9	12

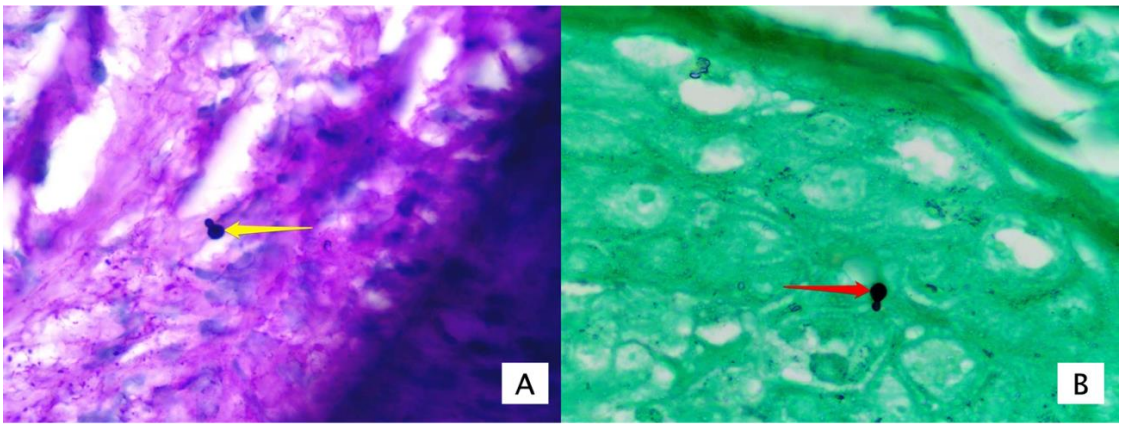

Fig. 1: Preparation of cervical cancer patients with periodic acid Schiff (PAS) staining – TR015 Squamous Cell Carcinoma (A) and Gomori methenamine silver (GMS) staining – TP148 Squamous Cell Carcinoma (B) at 1000 × magnification. The formation of yeast cells (budding) is shown on the tip of the blue-black arrow.

DISCUSSION

Cervical cancer is one type that has resulted in morbidity and mortality worldwide, including Indonesia [1, 4]. Until now, cervical cancer has experienced significant fluctuations, occupying the second position, contributing to new cases in Indonesia, and the third position, resulting in death [13]. Cervical cancer develops over time because of persistent infection with certain types of human papillomavirus (HPV). During its development, cervical cancer progresses through three main stages: precancerous changes characterized by changes in cells that experience abnormal development; an early stage characterized by the development of cancer cells limited to the cervix; and advanced stages characterized by constant changes in cells that have formed new tissue and produce more severe clinical manifestations, especially in the uterus, pelvic wall, or lymph nodes, as well as the rest of the body [21].

To date, advances related to screening, early detection, and diagnosis have continued to improve the accuracy of the results obtained and provide better treatment. As it progressed, advances in the confirmation of results, especially in the laboratory, have simultaneously made the diagnosis of viruses and other pathogens identifiable. This study aimed to reveal the presence of Candida fungi in slices of cervical cancer tissue from a hospital in Indonesia. According to the American Cancer Society [1], cervical cancer patients with histopathological diagnoses classified as decade 6 predominate the age decade; cervical cancer patients characteristically enter decade 6 (51–60 years). Thus, the diagnosis of cervical cancer and other pathogens was inhibited. It typically does not produce clinical manifestations that the patient can realize result from developing cancer cells that last a long time [22]. This corroborates previous research showing that the 6th and 7th decades of life account for approximately 2/3 of the patients in the Regional General Hospital of Kediri Regency, East Java, Indonesia.

Furthermore, based on the results of the histopathological diagnosis of the cervical biopsy samples, 81.25% of patients had cervical squamous cell carcinoma. These data are in line with reports from

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previous studies, which found this type of cervical cancer to reach 75% [6, 8]. The number of types of cervical squamous cell carcinoma is not surprising because anatomically, the cervix consists of two parts: the endocervix and the exo- or ectocervix [8]. The endocervix is a part of the uterus located inside, while the exocervix or ectocervix protrudes outward toward the vagina. The surface of the endocervix is lined with a continuous layer of mucosal cells on the surface of the uterine endometrial mucosa. In contrast, the exo/ectocervix surface is coated with a layered epithelial layer [10]. Additionally, the exocervix is in a setting with typical vaginal flora. This normal flora includes bacterial species such as Branhamella vaginalis and Lactobacillus, while fungi are *Candida albicans* species, and there are no viral microorganisms in the vagina [23, 24].

Certain human papillomavirus (HPV) serotypes are believed to be the cause of cervical cancer, and this HPV virus originates from the skin [25, 26]. For cervical cancer, this particular HPV serotype must be found and infect the basal cells of the cervical layered epithelial lining [2]. This can be realized if this particular serotype of HPV virus is facilitated in the basal layer of the cervical layered epithelium [18]. Among the microorganisms in the normal flora of the vagina, Candida fungi can invade tissues in their habitats. Based on the data obtained, periodic acid Schiff (PAS) staining detected Candida in eight cervical squamous cell carcinomas and one sample of cervical adenocarcinoma [27, 28]. This shows that Candida invasions are more prevalent in the exo/ectocervix, its normal habitat. Likewise, Gomori methenamine silver (GMS) detected Candida in ten cervical squamous cell carcinoma and two cervical adenocarcinoma samples. Owing to the cross-sectional study design, the fact that Candida fungi have been found in cervical cancer samples and cervical cancer tissue has not been able to prove that the presence and invasion of Candida in exo/ectocervical tissue is a risk factor for cervical cancer. However, current results provide updated information regarding the coexistence of Candida invasion in exo/ectocervical tissue and the incidence of cervical cancer [27, 29–32].

GMS staining detected more Candida than PAS staining did. Microscopic examination images showed that GMS staining was better than PAS staining due to the visible contrast. This is characterized by black structures in the green field compared with pink or bright structures in the red field [33]. Other researchers also recognize the ease of GMS staining in their publications, which has become the gold standard for the examination and staining of histopathological preparations [17, 34]. However, the compatibility between PAS and GMS staining was 84.4%. This number shows that the compatibility between the two dyes is strong according to Lin's concordance correlation coefficient. The presence of certain HPV serotypes in the vagina is very rare because HPV is not part of the normal vaginal flora. Changes in basal cells of the exo- or ectocervix surface-layered epithelium during the embryonic stage are the first signs of cervical cancer. The HPV virus must pass through sufficient defenses to enter the basal layer of the cervical-layered epithelium. This is possible when the integrity of the cervical layered epithelial layer is first damaged [25, 28, 35]. However, the presence of *Candida albicans* in the vaginal area gives certain HPV serotypes a chance to reach the basal layer of the exo/ectocervix. Thus, *Candida albicans* in cervical cancer tissue provides early evidence of the risk factors for cervical cancer [15, 23].

However, there is still limited literature on the presence of *Candida albicans* in the surface-layered epithelium of the exocervix as a risk factor for cervical cancer. Previous studies have revealed that the long-term use of contraceptive hormones, multipartner sexual intercourse, and alcoholism are major risk factors for cervical cancer [9]. Smith et al. [27] and Brandolt [29] reported that the prevalence of vulvovaginitis caused by Candida was 49.3%. In contrast to a previous study, this study obtained 13 samples (40.7%) from 32 samples examined for vulvovaginitis. Among Candida fungi, *Candida albicans* is the species that dominates up to 51.4% of nonpregnant women [29]. The prevalence rate of Candida vulvovaginitis increases to 90.38% in pregnant women [30]. Thus, detection of Candida species in cervical cancer tissue specimens has been demonstrated using GMS and PAS. Investigations related to the invasion of *Candida albicans* into the vagina should continue.

Research limitations

Although this study did not establish that Candida invasion of the cervix is a risk factor for cervical cancer, it is obvious that the presence of Candida in the cervical tissue under examination is proof that patients have Candida vulvovaginitis. This study did not identify any patient risk factors or clinical characteristics. This is because this study focused only on identifying the presence of Candida species in cervical cancer tissues using different colors.

CONCLUSION

The incidence of cervical cancer has developed and predominantly occurs at the age of 51–60 years, classified as decade 6. The results of the histopathological diagnosis of the cervical biopsy samples examined were as follows:81.25% had cervical

squamous cell carcinoma and 18.75% had cervical adenocarcinoma. The findings of this study show that staining with GMS can detect more Candida fungi than PAS staining. Microscopic examination proved that GMS staining was better than PAS staining because of the visible contrast of the black structures in the green field. However, this study did not show that Candida invasion of the cervix is a risk factor for cervical cancer. Further research is needed on the determinants that influence the presence or invasion of Candida into the cervix and the incidence of cervical cancer.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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AUTHOR'S CONTRIBUTION

AR: conceptualization, methodology, data collection, data analysis, manuscript preparation; RJS: Supervisor, data validation, results and data collection, manuscript finalization; APK: Validation of results, methodology development, manuscript preparation, manuscript finalization