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ORIGINAL ARTICLE

Prevalence of *Gardnerella vaginalis* infection and antibiotic resistance pattern of isolates of gynecology clinic patients at Shahriar Noor Hospital from January to June 2020 by PCR and culture methods

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ABSTRACT

Background and Objectives: *Gardnerella vaginalis* is one of the most important causes of prevalent genital infections that pose serious risks. This study aimed to determine the prevalence of *Gardnerella vaginalis* and antibiotic resistance pattern of isolates of patients referred to the gynecology clinic of Shahriar Noor Hospital by PCR and culture methods.

Materials and Methods: The study was conducted on 500 patients who had suffered from a vaginal infection. The demographic data of patients were studied. For diagnosis of *Gardnerella vaginalis* isolates, cultivation in anaerobic conditions, biochemical tests, PCR and *Gardnerella vaginalis* antibiotic susceptibility test to metronidazole and clindamycin were performed. Data analysis was performed utilizing SPSS statistical software version 19 and the Chi-square test.

Results: Among the 500 patients, 173 were diagnosed with *Gardnerella vaginitis.* There was a significant relationship between age group, level of education, and contraceptive method with *Gardnerella vaginosis* incidence. Performing antibiotic susceptibility tests showed that the resistance of *Gardnerella vaginalis* isolated strains to metronidazole and clindamycin was 86.12% and 17.34%, respectively.

Conclusion: The high prevalence of *Gardnerella vaginalis* infections confirms the critical role of the bacterium in the occurrence of bacterial vaginosis. Therefore, it is necessary to check the prevalence of bacterial infections to recommend the correct medical treatment in different societies.

Keywords: Antibiotic resistance; Bacterial vaginosis; *Gardnerella vaginalis*; Prevalence; Polymerase chain reaction

INTRODUCTION

Vaginitis is a general term for vaginal disorders caused by infection, inflammation, or normal vaginal flora changes. Bacterial vaginosis is one of the most important types of infectious vaginosis. The causes of nonspecific vaginal discharge or bacterial vaginosis are different, but the most common causes are anaerobic microorganisms. The normal vaginal flora is mainly composed of facultative anaerobic microorganisms such as *lactobacilli* (1). Several factors can alter the composition of the vaginal flora, including the patient's age, level of sexual activity, hormonal conditions, and health status (2). In bacterial vaginosis, the number of bacteria reaches 10^9-10^{11} cfu/ml, and its aerobic *lactobacilli* decrease, and

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anaerobic bacteria such as *Bacteroides ureolyticus, Gardnerella vaginalis, Atopobium vaginae,* and *Mycoplasma hominis* increase (3). *Gardnerella vaginalis* is a facultative anaerobic bacterium found in less than 1% of cases in the women's normal vaginal flora. *Gardnerella vaginalis* is a Gramnegative to Gram-variable *coccobacillus* with pleomorphic rods that are nonmotile and do not possess flagella, endospores, or typical capsules (4). It was first isolated and described by Leopold in 1953 from a cervical sample of women with cervicitis (5). The collaboration of this bacterium with anaerobic bacteria such as *Bacteroides* and *Mycoplasma* species in the vaginal mucosa causes bacterial vaginosis; therefore, *Gardnerella vaginalis* is called *Gardnerella* Associated Vaginosis (GAV) (5, 6). The disease is caused by a toxin with a molecular weight of 61-63 KDa that effects on erythrocytes (7). Among the problems caused by *Gardnerella vaginalis,* we can mention urinary tract infections (UTIs), pelvic inflammatory disease (PID), vaginal cuff cellulitis, chorioamnionitis, premature rupture of fetal membranes, premature labor, tubal infertility, cystitis, and abortion. In addition, Patients with GAV are at increased risk for vaginal infections because the levels of protective lactobacilli are reduced and inflammation is present. *Gardnerella vaginalis* is considered sexually transmitted diseases (STDs) (1, 5, 8). *Gardnerella vaginitis* is the most common bacterial vaginosis in reproductive ages. Accordingly, pregnant women who are suffering from this disease are considered as high-risk groups. Therefore, early diagnosis and treatment of vaginitis are important (9, 10). Two clinical diagnostic methods, Amsel and Nugent criteria, are usually used to diagnose bacterial vaginosis (11, 12). Metronidazole and clindamycin are commonly used to treat bacterial vaginosis. Both drugs are available as oral capsules and vaginal ointments (13, 14). In this regard, considering the hypothesis of high bacterial infection in the study population, the aim of this study was to investigate the infection coused by *Gardnerella vaginalis* and determine its pattern of antibiotic resistance in women referred to the gynecology clinic of Shahriar Noor hospital.

MATERIALS AND METHODS

Case selection. In this descriptive observational study, 500 women referred to Noor hospital gynecology clinic who had suffered from vaginal infection from January to June 2020 were examined. Patient records were collected by obtaining patients' consent and preparing questionnaires containing patient profiles such as patient age, referral reason, symptoms, number of deliveries, contraception methods, history of the previous infection, and use of antibiotics. Inclusion criteria included an increase in abnormal vaginal discharge with an unpleasant odor, inflammation and itching of the vagina, and dysuria. Exclusion criteria were the use of antibiotics and vaginal gels in the last 24 hours, having sexual contact in the last 24 hours, having gynecological surgery in recent days, and menstruation.

Sampling and diagnosis of bacterial vaginosis. A sterile speculum (without disinfectants) was inserted into the patient's vagina, then the posterior fornix of the vagina was sampled with three swaps. Swaps were used for Amsel criterion, microscopic observation, and culture. In order to diagnose bacterial vaginosis, four diagnostic criteria of Amsel were used, including examination of vaginal discharge, Gram staining, and direct observation of slides containing clue cells; the Whiff test was performed by adding 10% potassium hydroxide to a drop of vaginal discharge and inhaling the smell of amine and measuring the pH of vaginal discharge (11).

Culture and isolation of *Gardnerella vaginalis.* One of the swaps was cultured on Columbia Agar (Kulb / Canada) containing 5% human blood with a combination of 2 mg Gentamicin, 5 mg Nalidixic acid, and 1 mg Amphotericin B (manufactured by Haimedia / Mumbai, India). The plates were incubated in a 35°C incandescent anaerobic jar (5-10% CO2). Diagnostic tests including Oxidase, Catalase, Motility, Indole, Methyl red, Hippurate hydrolysis (Padtanteb Company / Iran), Nitrate reduction, Carbohydrate fermentation, Lysine decarboxylase, and Urease test were used to identify *Gardnerella vaginalis.*

Determination of antibiotic susceptibility by Epsilometer test (E-test). For this purpose, clindamycin and metronidazole E-test strips (manufactured by Liofilchem /Italy) were used. The E test was performed with Mueller-Hinton agar plates (diameter, 140 mm). In this method, after preparing the bacterial suspension according to the 0.5 McFarland standard turbidity, the plates were inoculated with the adjusted inoculum suspensions. E-test strips representing

the antibiotics metronidazole and clindamycin were placed on the agar and incubated at 37°C for 24 hours. Finally, by examining the plates, the minimum inhibitory concentration was read based on the intersection of the elliptical zone of growth inhibition with the MIC scale on the E-test strip. The susceptibility, resistance, and intermediate status of *Gardnerella vaginalis* to these antibiotics were determined.

Molecular detection of *Gardnerella vaginalis* isolates: bacterial genome extraction. Qiagen bacterial DNA extraction kit called QlAamp DNA Mini Kit (250) was used to extract the DNA of all *Gardnerella vaginalis* isolates. The absorbance ratio at 260 nm vs. 280 nm with a Spectrophotometer Nanodrop (USA DS-11, Denovix) was determined, and the quantity and quality of the extracted DNA were evaluated to assess the concentration and optical density of DNA.

Polymerase chain reaction method. The oligonucleotides, which were used as primers to detect the gene of *Gardnerella vaginalis*, are presented in Table 1. These sequences were verified using the blast section of the NCBI BLAST search tool database https://blast.ncbi.nlm.nih.gov/Blast.cgi to ensure the continuity of the produced primers. In brief, PCR was carried out in a total volume of 25 µl, containing 12.5 µl master mix, 5 µl DNA, 6.5 µl dd H2O and 0.5 µl (10 pmol) of each primer. A negative control (containing 1 µl of DNase/RNase-free water instead of genomic DNA) and positive control (containing 1 µl of genomic DNA from a pure culture of *Gardnerella vaginalis*) were included in each set of reactions. The tubes were placed in a thermocycler (Applied Biosystems, ABI-2720) that was programmed with the following protocol: an initial denaturation (94°C for 7 min), followed by 30 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s) and extension (72°C for 45 s), with a single final extension of 7 min at 72°C. After the PCR reaction, amplified products were analyzed in 1% agarose gel stained using Safe View (Qiagen. Co. IRAN). The electrophoresis was run for 30 min at 170 volts. Finally, according to the manufacturer's instructions, the results were visualized using the GelDoc (Bio-Rad) system. The PCR product's size was determined using a 100 bp DNA Ladder marker (Sinoclone/ Iran). These fragments were also sequenced by Royan Zistagene Company to ensure the correct amplification of the desired fragment and the accuracy of the products created in PCR.

Determination of PCR sensitivity. To perform PCR sensitivity test, from a sample with 1 µg/ml *Gardnerella vaginalis* DNA concentration , serial dilutions (100ng, 10ng, 1ng, 100pg, 10pg, 1pg, 100fg, 10fg, 1fg) were prepared and finally PCR test was performed on these samples.

Specificity of PCR. The specificity of PCR was determined using the extracted DNA of various bacteria, including *Staphylococcus epidermidis, Escherichia coli, Enterococcus faecalis,* and *Streptococcus agalactiae.*

Statistical analysis. The rates of infection with *Gardnerella vaginalis* in groups based on sociodemographic, medical, reproductive, behavioral, or microbiological variables were analyzed in SPSS software (version 19) using the K_2 test. A p-value <0.05 was used as a threshold for statistical significance.

Ethical statement. The above-mentioned sampling was approved by the Ethical Committee of Islamic Azad University of Karaj, Iran (ethical code: IR.IAU.K.REC.1399.017). Also, the informed consents were obtained from the subjects before participation in this study.

RESULTS

Results of sampling, culture, and isolation of *Gardnerella vaginalis* Among 500 women suspected of having bacterial ,173 cases were diagnosed with *Gardnerella vaginitis.* Examination of biochemical test results showed that hippurate hydrolysis, glucose, and maltose fermentation tests were positive and

Table 1. Primers used in this study

Primer Name	Oligo sequences 5'→3'	Size	Reference
Forward 16srRNA	5′TTACTGGTGTATCACTGTAAGG3′	331 bp	(14)
Reverse 16srRNA	5′CCGTCACAGGCTGAACAGT3′	331 bp	

negative reactions in all the other biochemical tests (Fig. 1).

Results of the determination of antibiotic susceptibility by E-test. The antibiotic susceptibility test results showed that 73.98% of isolates of *Gardnerella vaginalis* were sensitive to clindamycin, and 86.12% were resistant to metronidazole. The MIC results showed that the minimum inhibitory concentrations for the antibiotics clindamycin and metronidazole were respectively 0.64-0.94 and 1-1.5 µg/ml. (Fig. 2). Also, by comparing the obtained results with the table provided by the manufacturer of E-test strips (Italy - Liofilchem), the status of susceptibility, resistance, and intermediate of *Gardnerella vaginalis* bacteria to the antibiotics presented in Table 2 were determined.

Molecular detection results of *Gardnerella vaginalis*. After amplification of the 16SrRNA gene for all isolates of *Gardnerella vaginalis*, PCR product gave a specific band 331 bp with the allelic ladder as shown in Fig. 3.

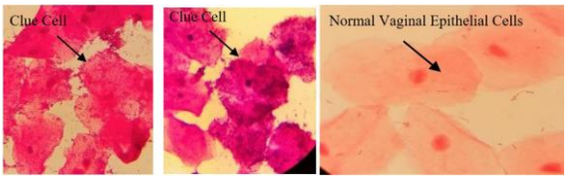

Fig. 1. Optical microscope image of Coccobacilli bacteria accumulating around vaginal epithelial cells compared to normal vaginal epithelial cells at 100× magnification

Fig. 2. Elliptical inhibition zone appears around the strips due to increased antibiotic concentration

Table 2. Frequency of minimum inhibitory concentration of *Gardnerella vaginalis* strains compared to clindamycin and metronidazole

Name of antibiotic	**Metronidazole**			**Clindamycin**		
MIC (µg/ml)	**(0.016-256)**			**(0.016-256)**		
Sensitivity status	S	R	I	S	R	I
	8≥	32≤	16	4 ≤	1-2	0.5≥
Number (percent)	16	149	8	128	30	15
	(9.24)	(86.12)	(4.62)	(73.98)	(17.34)	(8.67)

(S: Sensitive, R: Resistant, I: Intermediate)

PCR sensitivity results. The Sensitivity of the PCR method at the DNA level was determined to be 10fg (Fig. 3).

The high specificity of PCR was determined using four other important bacterial agents and found to be positive only for *Gardnerella vaginalis* (Fig. 3).

PCR products sequence analysis. The result of comparing the gene sequences obtained from this study with L08167.1 indicated that the sequences have 100% similarity, which indicates the accuracy of the PCR result. This complete similarity is also very important in terms of diagnosis.

The frequency of *Gardnerella vaginalis* in patients with bacterial vaginosis. This study showed that out of 500 vaginosis samples, 173 were diagnosed as *Gardnerella positive* samples, from which 38.72% were pregnant, and 61.27% were non-pregnant.

Descriptive analysis of demographic characteristics. In this study, patients with vaginitis were investigated in 7 age groups 15-20, 21-25, 26-30, 31-35, 36-40, 41-45, and 46 years and up. Also, education level and occupational conditions, marital status, Contraceptive method and pregnancy status of the patients were investigated, and, The results of the chi-square test were as follows: There was a higher rate of *Gardnerella vaginalis* infection in the age groups of 26-35 and 46 years and up (P <0.05). we observe a significant relationship between reducing the prevalence of GAV and increasing the level of education (P <0/025). There was no statistically significant difference between the rate of infection in groups with and without sexual activity. Also, there was no significant difference between marital status and Vaginosis caused by *Gardnerella vaginalis* (P>0.05).test results also showed the statistically higher prevalence of *Gardnerella vaginalis* in users of natural and hormonal contraceptive methods and IUD contraceptive method (P<0.01). The study reported the highest rate of GAV in non-pregnant women, and there were no statistically significant differences between the rate of infection and pregnancy status (P>0.05). Also, no statistically significant differences were observed

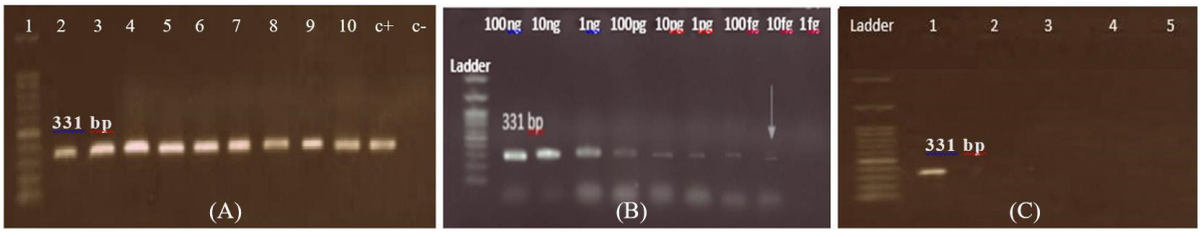

Fig. 3. (A) Agarose gel electrophoresis showing amplification of the 16S-rRNA gene for *Gardnerella vaginalis*, Lane 1: DNA marker (Ladder 100 bp), Lanes 2-10: DNA samples positive for 16S-rRNA gene, Lane 11: positive control Lane 12: negative control, (B) PCR susceptibility test using sequential dilutions of *Gardnerella vaginalis* DNA, (C) PCR specificity test, Lane 1: *Gardnerella vaginalis* DNA, Lane 2: *Staphylococcus epidermidis* DNA, Lane 3: *Escherichia coli* DNA, Lane 4: *Enterococcus faecalis* DNA, Lane 5: *Streptococcus agalactiae* DNA

between the number of deliveries and the occurrence of vaginosis caused by *Gardnerella vaginalis* (P>0.05).

DISCUSSION

According to the World Health Organization (WHO), three infectious agents, *Candida*, *Trichomonas*, and *Gardnerella*, are the main causes of vaginitis and 90% of vaginal infections. The incidence of bacterial vaginosis varies depending on the evaluated population (15). In the present study, the frequency and diagnosis of bacterial vaginosis connected with *Gardnerella vaginalis* and the isolation of this organism, its antibiotic resistance were investigated. Also, demographic data, fertility, and behavioral factors of women with vaginosis referred to the gynecology clinic of Shahriar Noor hospital were studied. The results show that out of 500 patients, 173 (34.6%) had *Gardnerella vaginalis* infection.

A study by Sabour et al. (2018) revealed that the overall prevalence of BV among Iranian women was reported to be 18.9% (16.5% of which non-pregnant women and 28% of which pregnant women) and *Gardnerella vaginalis* was the most prevalent isolated bacteria. (16). In the study by Mohammadzadeh et al. (2019), 27.4% of patients had BV, and *Gardnerella vaginalis* was found in 100% of women with BV and in 56.7% of women with normal vaginal discharge (17). In a study by Janulaitiene et al. (2017) 119 female vaginal specimens were studied which 29 samples were infected with bacterial vaginitis, and the prevalence of *Gardnerella* in them was reported to be 100% (18). In the study of patient's ages, the results showed a significant relationship between age groups and the possibility of infection with *Gardnerella vaginalis.* The highest prevalence was observed in 26-30, 31-35, and 46 years and above. The increase in infection caused by *Gardnerella vaginalis* in the ages of 26-30 and 31-35 indicates that *Gardnerella vaginalis* is sexually transmitted, and its prevalence is higher in sexually active people. Also, the abundance of GAV results in women over 46 years can be due to a significant decrease in the level of education and awareness, and physiological condition of the vagina. Gómez et al. (2019) described the vagina's physiological condition during menopause, suitable for vaginosis and indicated that the vaginal microbiota plays a key role in preventing colonization by pathogenic organisms and gynecologic female health (19). A study by Moris et al. (2001) on 8989 women showed that *Gardnerella vaginalis* as a bacterial vaginosis agent affects different age groups and is more common in the age group over 30 years, consistent with the present study (20). In the present study, a significant relationship was observed between increasing education and reducing GAV incidence. Also, a significant relationship was detected between increasing the level of education and reducing GAV incidence. Ahmadnia et al. (2016) stated that lower education level is associated with increased bacterial vaginosis incidences (21). However, most of the patients were in the middle and lower economic level, a more extensive study was required to examine the relationship between the disease and the level of education more closely. Our results showed that the isolation of *Gardnerella vaginalis* was associated with a decrease in the number of *lactobacilli.* Although the mechanism of this change in the microbial flora of the female vagina is unknown and the pathogenicity of *Gardnerella vaginalis* that reduces the number of lactobacilli has not yet been identified, this inverse relationship indicates that *Gardnerella vaginalis* can

be regarded as a pathogen and a causative agent of bacterial vaginosis (1, 8).

A study by Tsai et al. (2019) showed that the alteration of the normal vaginal flora and the reduction of lactobacilli increases the growth of anaerobic bacteria *Gardnerella vaginalis,* which causes vaginal secretion. (22). One of the parameters considered in this study was the contraceptive method. The results showed that the use of natural and hormonal methods increases the risk of *Gardnerella vaginalis* infection, and the use of IUD is recognized as a risk factor for GAV, which is consistent with the mechanism of action of this device, causing inflammation in the uterus. The results also showed that using a condom significantly reduced the risk of infection with *Gardnerella vaginalis.* Ahmadnia 's study (2016) on 274 married women and Cheraghi's study (2013) on 1448 non-pregnant women showed that the highest prevalence of *Gardnerella* infections was related to LD and IUD users, which consistent with the result of our study (21, 23). In a study by Amini et al. (2009), it was found that there was a significant relationship between the Usage of IUD and condom in the development of GAV. Using condoms was considered a protective method for vaginal infections and BV was diagnosed significantly more frequently in women with IUD (3), and conversely, the usage of IUD increases the risk of genital infections (2, 24). In a study by Ranjit et al. (2018), the prevalence of bacterial vaginosis was higher in those who used birth control pills (25).

After identifying and isolating *Gardnerella vaginalis* from patients, the antibiotic sensitivity of the isolated strains was measured by the MIC (test-E) method, and the results are shown in Table 2. As can be seen, there was antibiotic resistance to metronidazole in 12.86% of patients with GAV and antibiotic resistance to clindamycin in 34.17% of patients. Therefore, since the cases of drug resistance to metronidazole are more than to clindamycin, it is better to use clindamycin for the treatment of GAV. Moreover, culture in a specific media and antibiotic susceptibility test must be performed in case of bacterial vaginosis caused by *Gardnerella* due to the observed antibiotic resistance in some isolates against the two antibiotics, metronidazole and clindamycin. If there is resistance to either of these two common antibiotics, an alternative and effective treatment method should be replaced. Nagaraja's study (2008) showed that 68% of all isolates were resistant to metronidazole while 76% were sensitive to clindamycin, and Clindamycin therapy has better clinical efficacy than metronidazole in cases of recurrent bacterial vaginosis (26). A study by Hakimi et al. (2018) on 607 women and Decena et al. (2006) on 90 women showed that adjuvant treatment using prebiotic vaginal gel increased the efficacy of treatment of BV with oral metronidazole. (27, 28). According to another study (2002), 29% of *Gardnerella vaginalis* strains were resistant to metronidazole. Recent studies shown that clindamycin is more effective than metronidazole against *Gardnerella vaginalis* (3). In a study by Bostwick et al. (2016), it was shown that in the case of BV, the most commonly prescribed antibiotics are clindamycin and metronidazole, and resistance to these compounds is increasingly common (29). Resistance to this antibiotic may be due to the widespread use of this drug to treat bacterial vaginosis. Comparing the results of PCR and Amsel methods showed that PCR has higher sensitivity than Amsel criteria. Despite this, due to the relatively limited facilities in the country's clinical centers, the Amsel method is preferable to PCR for rapid diagnosis and treatment of this disease by obstetricians and gynecologists. Also, molecular methods were more efficient compared to culture to detect *Gardnerella vaginalis* because the culture method is time-consuming and requires a lot of effort. Additionally, sensitivity and specificity tests were performed for this molecular method to check the accuracy of the PCR test. The results showed that the primers used in this study binding to the lowest available amounts of *Gardnerella vaginalis* DNA (10fg), indicating a high level of sensitivity (Fig. 3). Also, in the results of determining the specificity of the PCR test, it was observed that the primers only bind to *Gardnerella vaginalis* genomic DNA, so the specificity of the test was reported 100% (Fig. 3). A collection of reviews in STDs centers (1979) showed the prevalence of the bacterium in university clinic centers, which indicates that *Gardnerella vaginalis* is sexually transmitted and is more prevalent in sexually active people. Most infected people are in the age group of 21-25 years 30.8% of non-pregnant women and 23.3% of pregnant women) (30).

CONCLUSION

Based on the results obtained in this study, it was found that *Gardnerella vaginalis* is present in a

high percentage of patients with bacterial vaginosis, which confirms that *Gardnerella vaginalis* is one of the important bacterial agents in the occurrence of bacterial vaginosis. Therefore, due to the importance of this disease, the abundance of this bacterium in vaginal secretions, its complications and antibiotic resistance of some strains of *Gardnerella vaginalis* to conventional antibiotics, determine the phenotypic and genotypic characteristics of *Gardnerella vaginalis* to better identification, isolation, and treatment of this pathogen in different communities is necessary. Since limited access to medical services, low level of awareness, and cultural barriers are the causes of delayed treatment of this disease, to control and prevent vaginal infections and their complications, it is recommended that women receive the necessary training on health issues. In cases where there is a possibility of infection caused by *Gardnerella vaginalis* or if the patient has symptoms or laboratory methods show the incidence of this disease, it is recommended that cultivation of *Gardnerella vaginalis* be performed in specific media to determine the antibiotic susceptibility of the bacterium and in cases of observed drug resistance to using a practical and alternative medicine. Also, study of environmental factors affecting vaginal infection caused by *Gardnerella vaginalis*, determining the Gardnerella vaginalis biotypes, and evaluating the antibiotic resistance virulence factors of *Gardnerella vaginalis* strains are recommended to identify *Gardnerella vaginalis* infection thoroughly.

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