
Molecular prevalence of *Trichomonas vaginalis*, *Treponema pallidum* and *Neisseria gonorrhoeae* in self-collected cervicovaginal samples from riverside women, Amazonas, Brazil

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Abstract

Introduction: Sexually transmitted infections are an important Public Health problem, because if not diagnosed and treated correctly they can result in severe complications, such as cervical cancer, miscarriages, infertility, premature birth, increased risk of transmission of HIV, among others. In remote places, such as the riverside communities of the Brazilian Amazon, the diagnosis and treatment of these infections are problems that are still difficult to overcome.

Objectives: This study aims to determine the prevalence of three sexually transmitted pathogens - *Trichomonas vaginalis*, *Treponema pallidum* and *Neisseria*

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gonorrhoeae - in self-collected samples from riverine women and to correlate prevalence rates with sociodemographic, clinical and behavioral characteristics.

Methodology: Cervico-vaginal samples obtained through self-collection with the COARI® (KOLPLAST®) brush from 420 women living in rural communities in Coari city, Amazonas, Brazil were analyzed. DNA extraction was performed with ReliaPrep™ Kit (Promega®) and pathogen detection was performed using the multiplex PCR technique.

Results: The epidemiological and behavioral data showed that the age of the participants ranged between 18 and 70 years, with a mean age of 34 years (SD=12); the majority (90.1%) work in agriculture, with a family income of less than one minimum wage (88.1%) and a low level of schooling. The mean age of first sexual intercourse was 15.4 (SD=2.2) and 83.1% had 1 to 5 sexual partners throughout their lives. The results of detection by multiplex PCR indicated a prevalence of 1.9% for *T. vaginalis* among the investigated women; *T. pallidum* and *N. gonorrhoeae* were not detected in the analyzed samples.

Keywords: Sexually Transmitted Infections. Amazon. Multiplex PCR. *T. vaginalis*. *T. pallidum*. *N. gonorrhoeae*.

INTRODUCTION

Sexually Transmitted Infections (STIs) are a serious Public Health problem, especially for low-income people living in developing countries. Such infections lead to an increase in health care expenses, both directly for diagnosis and treatment, and for the management of the various consequences arising from these infections, such as pelvic inflammatory disease (PID), infertility, cervical cancer, abortions, prematurity, low birth weight, increased risk of HIV transmission, among others (Van Gerwen and Muzny 2019; Dias et al. 2021; Smolarczyk et al. 2021; Bonab et al. 2021). These infections can also interfere negatively in the social life of these people, impairing their affective/sexual relationships, interfering with patients' contact with society and reducing their workforce.

Trichomoniasis, caused by the protozoan *Trichomonas vaginalis*, is one of the most prevalent non-viral STIs worldwide (Van Gerwen and Muzny 2019). However, our understanding of the epidemiology of *T. vaginalis* infection here in Brazil contains gaps, as this infection has not been evaluated in large population-based studies and the disease is not normally monitored by public health agencies (Rocha, Azevedo, et al. 2019). In addition, the infection can be asymptomatic, making it difficult to identify infected people. In women, when symptomatic, the most common symptoms are dysuria,

itching and foul-smelling vaginal discharge; severe inflammation in the cervix is also common (Van Gerwen and Muzny 2019; Leitsch 2021).

Infection with the bacterium *Neisseria gonorrhoeae* is usually asymptomatic in women. But, despite its asymptomatic character, if not diagnosed and treated properly, it can cause ascending infection in the female genital tract, which can lead to PID, infertility, endometritis, chronic pelvic pain and ectopic pregnancy (Springer and Salen 2021; Whelan et al. 2021). Additionally, in the long term, untreated infection can lead to arthritis and infective endocarditis (Benavent Núñez et al. 2019). Vertical transmission may be associated with low birth weight and neonatal conjunctivitis, which can progress to blindness (Whelan et al. 2021).

Another highly relevant sexually transmitted bacterium is *Treponema pallidum*, the causative agent of syphilis. This disease has a natural course with manifestations in stages: primary, secondary, and tertiary syphilis, in addition to latency periods. Clinical manifestations at different stages involve genital ulcers and lymphadenopathy (primary syphilis); fever, headache, maculopapular rash on neck, arms, chest, back, palm and sole (secondary syphilis); and cardiac, neurological, and destructive skin lesions (syphilitic gums) (tertiary syphilis), although this stage is no longer as common. The infection can also be latent, that is, without the manifestation of signs and symptoms (Peeling et al. 2017; Lasagabaster and Guerra 2019; Mercuri et al. 2022). One of the main problems of this infection is vertical transmission, as congenital syphilis can be particularly aggressive to the fetus, causing prematurity, rhinitis, jaundice, eye lesions, pneumonia, nephrotic syndrome, bone pain, abnormal teeth, among other early or late manifestations (Peeling et al. 2017; Keuning et al. 2020).

In the state of Amazonas, especially in the countryside, we found few epidemiological studies on infections caused by sexually transmitted pathogens, so knowledge of the prevalence and incidence of these infections is deficient. The Brazilian Amazon is a region of great geographic dispersion, which, combined with its continental dimensions and the precarious socioeconomic conditions of the population, makes it difficult for riverine people to access adequate health services (Gama et al. 2018; Torres et al. 2018; Rocha, Moraes, et al. 2019; Rocha, Azevedo, et al. 2019). Given this context, this study aims to estimate the prevalence of three sexually transmitted pathogens - *T. vaginalis*, *T. pallidum* and *N. gonorrhoeae* - in samples collected from women living in rural areas of the Coari city, in Amazonas, Brazil.

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METHODOLOGY

Study design, population and sample - This is a cross-sectional descriptive epidemiological study carried out with women living in the rural area of the Coari city in Amazonas, Brazil. Coari (Figure 01), is located 363 km from the city of Manaus (Capital of Amazonas), in the region of the Middle Solimões River, which can only be accessed by air and river. The estimated population was 84,272 inhabitants in 2018 (IBGE - Instituto Brasileiro de Geografia e Estatística 2018), with 405 communities in the rural area, distributed in 7 river channels. The communities included in our study are in the Upper Solimões River, Middle Solimões River, Lower Solimões River, Mamiá Lake, Copeá River, Coari lake and Ariá Island regions.

Considering the prevalence of STIs of 42% in general (Brasil. et al. 2008), with a margin of error of 5% and confidence of 95%, an “n” sample of 385 women was estimated. Inclusion criteria were being sexually active and being over 18 years of age. Women who were pregnant, menstruating and who had used vaginal ointments in the last 5 days were not included.



Figure 1. Geographic location of Coari city, Amazonas, Brazil.

SOURCE: Google (Adapted by Reis 2017).

Collection of data and biological samples - Initially, all women from the visited communities were invited to participate in the research. However, only those who met the inclusion criteria and agreed to participate in the research signed a Free and Informed Consent Term (FICT). They received instructions regarding the use of the COARI® brush (Kolplast®) and completed a standard questionnaire containing clinical, socioeconomic and behavioral questions. The participants carried out the collection in a private environment (bedroom

or bathroom) and then handed the collection device to the researchers, who identified and sealed the package immediately. The samples were stored in the Genetics and Molecular Biology Laboratory of the Federal University of Amazonas, in Coari city.

Laboratory processing - The bristles of the brushes were cut with a sterile scalpel blade and placed in a lysis solution containing proteinase K and Tris-EDTA buffer. DNA was then extracted using the ReliaPrep™ kit (Promega®) following the manufacturer's instructions. The verification of the efficiency and quality of DNA extraction was performed by amplifying human genomic DNA using the PCO3/PCO4 primer pair that amplifies a 281 base pair (bp) fragment of the human β -globin gene (Saiki et al. 1992). The reaction components were: 2.5mM dNTP, 0.8mM MgCl₂, 10x buffer, 5Mm of each primer, Platinum Taq DNA polymerase (INVITROGEN®) 0.2U/ μ L, 5.0ml DNA and water to complete the final volume of 25.0 μ l. The reaction was carried out in a Veriti® Thermocycler (Applied® Biosystems). Thermocycling temperatures and times were: 94°C for 30 seconds, 40 cycles of 94°C for 30 seconds, 58°C for 90 seconds and 68°C for 120 seconds, followed by 68°C for 5 minutes. In all PCR negative and positive controls were used.

The investigation of the DNA of the three pathogens in the cervicovaginal samples was performed by means of multiplex PCR (mPCR) with primers designed specifically for this study. To analyze the presence of *T. vaginalis*, a pair of primers TV01F and TV02R was developed, which amplifies 90 bp of DNA from the beta-tubulin gene; the detection of *N. gonorrhoeae* was performed with the pair of primers NG01Fe NG02R, which amplify a 140 bp sequence of the *porA* pseudogene and to investigate the presence of *T. pallidum*, the pair of primers TP01 and TP02 was developed, which amplify 194 bp of a conserved sequence of the *polA* gene (Table 01). As a positive control for *T. vaginalis* and *N. gonorrhoeae*, positive samples were used in the cell culture and for *T. pallidum*, a DNA sample extracted from the ulcer lesion (primary syphilis). The mPCR mix was prepared in a final volume of 25 μ L, containing: 2x PCR buffer, DNA, 2.5mM dNTP, 5mM each primer, 50mM MgCl₂ and 1U of Platinum Taq DNA polymerase (Invitrogen, Brazil) and water. The reactions were performed in a thermocycler (Applied® Biosystems).

The PCR products were separated by electrophoresis in a 1.5% agarose gel, stained in ethidium bromide, analyzed in ultraviolet light (UV) and photodocuments in a transilluminator device (Loccus® Biotechnology).

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Table 01- Sequences of primers designed for the study.

Pathogen	Primer	Sequence	Amplicon
<i>T. vaginalis</i>	TV01F	5'-CATTGATAACGAAGCTCTTTACG-3'	90 pb
	TV02R	5'- ATGGAACAAGGTGGTTAAGA-3'	
<i>N. gonorrhoeae</i>	NG01F	5'-TGTATTATGCCGGTCTGAATTA-3'	140 pb
	NG02R	5'-TTTCAATGGATCGGTATCACT-3'	
<i>T. pallidum</i>	TP01	5'-AAACGATACATTACAAATGACAGA-3'	194 pb
	TP02	5'-GCATAACATCCTGCCTAAGA-3'	

Statistical analysis - Data were tabulated and analyzed using SPSS version 2.0 for Windows. The results are presented in tables, in the form of absolute and relative frequency and/or mean or median. The analysis of difference in proportions and analysis of association or not between the variables was performed using Pearson's chi-square test (χ^2) and/or Fisher's test for cases in which the frequency was lower a ($p < 0.05$). The level of significance established in the application of the tests was 5%, with a confidence interval of 95%.

Ethical aspects - This research was carried out following all ethical issues related to research involving human beings, having been approved by the Research Ethics Committee of the Federal University of Amazonas (CAAE 62552716.1.0000.5020).

RESULTS

Sociodemographic, clinical and behavioral characteristics - 78 riverside communities in the city of Coari were visited, making a total of 420 women who accepted to be part of the study and were within the inclusion criteria. Table 02 describes the microregions, the number of communities and the amount of self-collected samples in each community.

Table 02. Identification of the micro-region, riverside communities and self-collected samples. Coari, 2022.

MICRO-REGIONS	NUMBER OF COMMUNITIES	N	%
Upper Solimões River	15	66	15,7
Middle Solimões River	8	78	18,5
Lower Solimões River	1	14	3,3
Mamiá Lake	15	96	22,8
Copeá River	26	114	27,1
Coari Lake	12	51	12,1
Ariá Island	1	1	0,2
Total	78	420	100

The age of the participants ranged from 18 to 70 years, with a mean age of 34 years (SD=12). The level of education of these women was low, as 49% of them

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had not even completed elementary school. Most of them (93.4%) reported working, with agriculture as their main occupation (90.1%). The family income of these women was very low (88.1% had a family income of less than 1 minimum wage – R\$937.00 in 2018 – about \$180). Regarding marital status, 85.3% of the participants reported being married or living in a stable union with a partner. Table 03 provides detailed information on the demographic characteristics of the sample under study.

Table 03 - Description according to the frequency of the sociodemographic characteristics of the women participating in the study. Coari, 2022.

Variables (n= 420)	F	%
Age		
18 --- 25	112	26,6
25 --- 35	127	30,2
35 --- 45	88	20,9
45 --- 55	59	14,9
55 --- 65	32	7,6
≥ 70	2	0,4
Media ± SD	34 ± 12	
Amplitude	18 - 70	
Education		
Unschooling	30	7,1
Incomplete Elementary School	206	49,0
Complete Elementary School	93	22,1
Complete High school	83	19,7
Complete Higher Education	8	1,9
Work (job)		
Yes	392	93,4
No	28	6,6
Occupation (n= 392)		
Fisherwoman	10	2,6
Farmer	353	90,1
Small trader	2	0,5
Maid	1	0,2
Public Employee	21	5,3
Retired	5	1,3
Family income *		
Less than minimum wage	370	88,1
One minimum wage	43	10,2
Two minimum wage	7	1,7
Marital Status		
Single	42	10
Married/Stable union	358	85,3
Divorced/Separated/Widow	20	4,7

F= simple absolute frequency; SD= standard deviation; * Minimum wage in 2018: R\$ 937,00 (about \$ 180.00)

The clinical history of the women revealed that the mean number of pregnancies was 4.7 (SD=3.4) children. The mean age at first pregnancy of

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women (n=404) who had children was 16.2 (SD=4.4) years. Of the 420 participating women, 116 (27.6%) reported having had a miscarriage experience. 4.1% of participants reported having had a diagnosis of sexually transmitted infection in the past; most women (74.7%) reported gynecological clinical complaints at the time of collection, mainly pelvic pain and discharge. Table 04 provides detailed information on the clinical characteristics of these women.

Table 04 - Description according to the frequency of clinical characteristics of women participating in the study. Coari, 2022.

Variables (n= 420)	F	%
Pregnancy Number		
Media ± SD	4,7 ± 3,4	
Amplitude	0 – 20	
Age of 1st Pregnancy (n= 404)		
Media ± SD	16,2 ± 4,4	
Amplitude	12 – 32	
Number of Miscarriages (n= 116)		
One	93	80,2
Two	15	12,9
Three or more	8	6,9
STIs in the past		
Yes	17	4,1
No	375	89,2
Never had an STI test	28	6,7
Clinical Complaints (n= 314)		
Pelvic pain	193	61,5
discharge	155	49,4
Dysuria	110	35,0
Itching	110	35,0
Pain after sexual intercourse	93	29,6
Bleeding after sexual intercourse	10	3,2
Genital warts	6	1,9
Genital ulcers	6	1,9
Others	7	2,2

F= simple absolute frequency; SD= standard deviation

The data obtained regarding the sexual behavior of the participants showed that the mean age of the first sexual intercourse was 15.3 years (SD= 2.5) and the majority (83.1%) reported having had up to 5 sexual partners during their lifetime. When asked about the number of current partners, the majority reported having 1 steady partner (93.1%), while few women reported being without partners or having occasional partners. Of these women who reported having only one steady sexual partner, 65.7% said they did not use condoms. More detailed information about the sexual behavior of the participants can be found in Table 05.

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Table 05 - Description according to the frequency of the behavioral characteristics of the women participating in the study. Coari, 2022.

Variables (n= 420)	F	%
Age of First Sexual Intercourse		
Media ± SD		15,3 ± 2,5
Amplitude		9-29
Lifetime Partner Numbers		
1 to 5	349	83,1
6 to 10	59	14
Above 10	12	2,9
Current sexual partnership		
No partner	24	5,7
One partner	391	93,1
More than one partner	1	0,2
Only occasional partners	4	1,0
Use of Condoms with a Fixed Partner		
Yes	132	31,4
No	276	65,7
No fixed partner	12	2,9

F= simple absolute frequency; SD= standard deviation

Prevalence of STIs by mPCR - DNA detection of the three pathogens by mPCR, as proposed, showed that 8 women (1.9%) had *T. vaginalis* DNA. *N. gonorrhoeae* and *T. pallidum* were not detected, although all positive controls successfully amplified in mPCRs. Due to the low prevalence of infected women, little can be inferred about the characteristics of the infected group. Data related to *T. vaginalis* infection were crossed with sociodemographic, clinical and behavioral data, but no association was statistically significant.

DISCUSSION

The Brazilian Amazon region is characterized by being an area of continental dimensions and high demographic dispersion, with people living in communities located on the banks of rivers in small communities where it sometimes takes a few days (or even weeks) to reach the nearest city. These geographical peculiarities (a region “between rivers”) impose a lifestyle away from large urban centers and more structured health services. Even simple tests - such as rapid tests for STIs and the Pap smear - are difficult to offer these women (6.7% of the women in our study had never had any type of STI test!). Rapid tests for screening for syphilis, HIV and HBV can be easily performed by a simple finger prick, but for other pathogens, other collection and detection techniques have been needed. Cervico-vaginal self-collection devices have been studied in these regions as a means of facilitating the acquisition of biological samples for later detection - by rapid or molecular

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tests - of pathogenic microorganisms, such as HPV, *C. trachomatis* and *T. vaginalis* (Torres et al. 2018; Rocha, Azevedo, et al. 2019; Rocha, Moraes, et al. 2019; Rodrigues et al. 2019).

In the present study, we used the mPCR technique to verify the prevalence of infections by *T. vaginalis*, *N. gonorrhoeae* and *T. pallidum* in biological samples of exfoliated/sloughed cells in the bristles of the Coari® self-collection brush (Kolplast®, Brazil) of women living in the rural area of the city of Coari, in Amazonas. Research has shown that these self-collection devices are very promising for use in populations such as the one in this study, as they are generally well accepted by the participants, as in addition to providing more privacy and less discomfort at the time of collection, they save time and money spent on scheduling. and travel to health centers. In addition, they allow tracking of infected but completely asymptomatic women, and have shown sensitivity comparable to the biological sample collected by a professional. On the other hand, they bring as a disadvantage the fact that women do not receive an adequate pelvic exam for direct visualization of lesions by the professional (Lockhart et al. 2018; Rocha, Azevedo, et al. 2019; Camara et al. 2021). The device used in this study proved to be efficient for obtaining cervicovaginal cells, as we obtained 100% positivity when we performed the molecular detection of the b-globin gene in human DNA.

For molecular detection of microorganisms, in this study, we chose to perform multiplexing, as it has the advantage of detecting more than one pathogen in the same reaction, reducing time and expense of reagents. The designed primers were tested with a variety of sample types that were proven positive using other classic primers. For the detection of *T. vaginalis*, the TV01F/TV02R primers amplified a 90bp sequence of the beta-tubulin gene. This same gene was used in the design of the BTUB2/BTUB9 primers in the classic study by Madico et al., (1998), tested and validated in other studies (Crucitti et al. 2003) and has been used in Brazilian studies (Rocha et al. 2014; Costa-Lira et al. 2017).

We found in our study that 1.9% of women were infected with *T. vaginalis*. All infected women had clinical complaints, which were: discharge, itching, pelvic pain and bleeding after sexual intercourse (data not shown), which is in agreement with the literature (Leitsch 2021; Van Gerwen and Muzny 2019). The prevalence found in this study can be considered low in relation to other studies carried out in this region of Brazil. In a previous study, also using a self-collection device, the Evalyn® Brush (Rovers® Medical Devices), a higher positivity (5.7%) was also found in riverside women in the Amazon (Rocha, Azevedo, et al. 2019). In the urban area of the same

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city (Coari), but using a sample collected by a professional during a routine gynecological examination, a positivity of 12.7% was detected for *T. vaginalis* (Rocha et al. 2014).

The interesting study also carried out with Amazonian women (Rio Tapajós, Pará) by Rodrigues et al (2019) compared the detection rate of *T. vaginalis* in HIV-infected and non-infected women, also using a self-collection device. The authors found a low prevalence of *T. vaginalis* in uninfected women (0.9%) and a considerable prevalence in HIV-infected women (9.8%). Studies show that *T. vaginalis* infection increases the risk of HIV contamination, as it causes damage to epithelial membranes (micro hemorrhages) and also recruits defense cells that are targets for HIV; and the opposite is also true: HIV positive women are more likely to acquire and spread *T. vaginalis* (Masha et al. 2019). In our study, we did not assess HIV infection in women, either through examinations or with a specific question in the questionnaire, which is a limitation of this study.

For the detection of gonococcal DNA, we designed the primers NG01F and NG02R, whose target is the *porA* pseudogene, which encodes bacterial membrane proteins. This gene is expressed only in *N. meningitidis* and *N. gonorrhoeae*, but not in saprophytic species. Although this gene has homologous regions in these two species, the primers are designed in specific non-homologous regions. Primers that delimit a region of the *porA* pseudogene have been frequently used in prevalence studies in Brazil and worldwide (Rocha et al. 2014; Liu et al. 2017). In our study there was no positive sample for *N. gonorrhoea*, although in all reactions the positive control has amplified perfectly. It could be considered that a self-collected sample is not as suitable for analyzing the presence of *N. gonorrhoeae* DNA by nucleic acid amplification methods as a sample collected by professionals, generating false negatives. However, studies show high levels of agreement between the two types of samples (Arias et al. 2016; Galvez et al. 2021).

The low prevalence found in our study agrees with other studies carried out in urban-rural Amazonian regions, which also showed a low prevalence of *N. gonorrhoeae*. In the study by Rocha et al (2014) with women living in the urban area of the same city under study, Coari, the authors found 1.4% positivity in cervicovaginal samples from women undergoing routine gynecological examination. Benzakén et al (2012) found only 0.3% of women infected in the triple borderland region (Brazil, Colombia and Peru) of the Upper Solimões River. Rodrigues et al (2019) found a 2.4% prevalence in women in Pará, but in women living with HIV, the prevalence found by these authors was slightly higher: 4.9%. Galvez et al (2021), studying women in

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urban-rural Peruvian areas similar to ours, also did not find positive women for *N. gonorrhoeae* either in self-collected samples or in samples collected by professionals, and using commercial PCR kits.

Similarly, no positive samples were found for *T. pallidum* among the women evaluated in this study. The primers used (TP01 and TP02) flank a region of the bacteria *polA* gene, amplifying a 194bp fragment. It is not common to find epidemiological studies using only samples from vaginal fluids and vaginal exfoliated cells to detect this bacterium, because syphilis is a systemic infection, and treponema enters the bloodstream soon after the outcome of the initial stage of infection (primary syphilis). In the primary phase of the disease, there is a large amount of bacteria in genital ulcers - usually located on the labia majora, labia minora and perineum - and the diagnosis by PCR is quite feasible in this active phase of the disease. However, in the secondary and latent stages of the disease, serological tests are well established as the tests of choice for screening (Peeling et al. 2017; Lasagabaster and Guerra 2019; Çakmak et al. 2019).

Our sample of women studied had sociodemographic, clinical and behavioral characteristics that are quite common in riverside populations in general: low schooling, work predominantly in agriculture, low income, young age at first sexual intercourse, young age at first pregnancy and number of children well above of the national average (Gama et al. 2018; SEADE. Sistema Estadual de Análise de Dados. 2021). As for the marital status, we also found that the majority live in a stable union/marriage with a steady partner, with low adherence to condom use. Despite the low prevalence of infection by these pathogens found in this research, the data regarding the large number of women (74.8%) reporting clinical complaints suggestive of STIs, such as pelvic pain, discharge, dysuria, pruritus, pain after sexual intercourse, among others, and the expressive number of women who experienced spontaneous abortion (27.6%), suggesting that other sexually transmitted pathogens should also be investigated.

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