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Supplementary Materials

Prevalence and Risk Factors of Chlamydia trachomatis and Neisseria gonorrhoeae in University Students: A Study from Colombia

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Methodology

The sample size was calculated with the Epidat 4.2 program; considering previous studies, a prevalence of 7% was anticipated for the two infections, a confidence interval of 95%, a precision of 3%, and a design effect of 1. The results of this calculation estimated a sample of n=200. The instrument was developed based on scientific literature and validated through pilot tests and the test-retest technique with a sample of 31 students not included in the prevalence study. For quantitative variables, such as age, age at sexual initiation, and number of sexual partners, the Intraclass Correlation Coefficient was between 0.84 and 0.99. For qualitative variables, the percent agreement ranged from 57.1% to 100%, with less agreement on questions about knowledge and condom use in the last year and perfect agreement on questions about gynecological history and type of sexual partnerships. In addition, 64.8% of the questions had a Cohen's Kappa Index more significant than 0.6. Questions related to symptoms in the last year and knowledge had a Cohen's Kappa Index less than 0.6. Considering these results, the questions about symptoms in the previous year were removed from the final survey applied. For each variable, absolute and relative frequencies and the prevalence ratio (PR) were calculated without considering the participants who did not answer the specific question.

Likewise, in men, urine samples were collected by self-collection, taking the precaution of collecting from the first part of urination, preferably the first of the day or after four hours of retention. A self-collection device

was developed and validated for the female sample[1]. The specimens were transported in a refrigerated container at 6-8°C to the Universidad de Santander's Biomedical and Biotechnological Research Laboratory (LIBB) for pre-treatment in less than six hours.

Pre-treatment

The urine sample was centrifuged (3500 rpm x 10 min) in 15 mL RNA-free tubes, the supernatant was discarded, and the pellet was washed twice with 1 mL phosphate-buffered saline (PBS 1X), finally resuspended in 200 µL PBS 1X and stored at -80°C until DNA extraction; the remaining urine sample underwent the same pre-treatment and was stored as a counter sample. The vaginal sample was suspended in 600 µL of PBS 1X, 200 µL was collected and stored at -80°C for subsequent DNA extraction, and the remaining 400 µL was stored as a counter sample.

DNA extraction

According to the manufacturer's recommendations, DNA was extracted using a commercial Invisorb Spin Universal Kit (Invitek Molecular Cat No. D-13125). The concentration of the extracted DNA was evaluated by spectrometry using a NanoDrop 2000C (Thermo Fisher Scientific). The 260/280 ratio was used to indicate the sample purity. The DNA obtained was stored at -80°C until processing by end-point PCR.

Polymerase Chain Reaction - PCR

CT and NG were detected by end-point PCR, and primers directed against two specific genes in each bacterium were used (Table S1). For CT, primers directed at the gene coding for the major outer membrane protein (*omp*) were designed, considering the conserved regions of four gene variants (*omp1*, *ompA*, *ompB*, and *ompC*) found in different strains. Similarly, primers directed to the Cryptic Plasmid present in different serovars were designed. The primers were located in the ORF-3 region because this region is highly conserved. Regarding NG, primers were designed against the pseudogene *porA*, considering the presence of this gene in different strains. Likewise, primers were designed for the opa gene, considering the *opa* gene sequences (*opaB-opaK*) found in strain MS11. It is essential to mention that these primers concurred with the primers published by Priyadarshi et al.[2]. The sequences were obtained from the National Center for Biotechnology Information (NCBI). As an internal amplification control, the constitutive gene of human beta-globin (HBB) was used to evaluate the presence of inhibitors in the reaction[3]. Primers were validated by bioinformatics analysis using Primer3 software (v.0.4.0 at http://primer3.ut.ee), and specific alignments were verified using Primer-BLAST, the NCBI online tool (https://www.ncbi.nlm. nih.gov/tools/primer-blast/).

The commercial kit OneTaq Hot Start 2X Master Mix with Standard Buffer (New England Biolabs NEB Cat No. M0484) was used for PCR assembly following the manufacturer's recommendations. The PCR mix contained 1X OneTaq Hot Start Master Mix, 0.5 µM of each primer, and < 1000 ng of DNA in a final volume of 25µL. Amplification was performed on a ProFlex PCR System thermal cycler (Applied Biosystems), with PCR cycling conditions optimized for the detection of each gene. AMPLIRUN (VIRCELL S.L, Spain) CT (MBC012-R) was used as a positive control for CT, and NG extracted DNA from strain ATCC 19424; molecular grade water was used as a negative control. The amplified PCR products were evaluated by agarose gel electrophoresis (1.4% - 1.7%) stained with HydraGreenTM Safe DNA Dye, and the gels were observed in a MiniBis photodocumenter (DNR Bio-Imaging System).

PCR specificity

The specificity of PCR for CT and NG genes was evaluated using DNA from the eight most relevant uropathogens affecting human health[4], including *E. coli* (ATCC 10536), *K.pneumoniae* (ATCC 10031), *S. saprophyticus* (ATCC 15306, *E. faecalis* (ATCC 14506), *P. vulgaris* (ATCC 33420), *P. mirabillis* (ATCC 12453), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25913). Microorganisms were grown on Luria-Bertani agar (OXOID) for 24 h at 37°C. DNA extraction and PCR for each gene were performed using the abovementioned methodology. Analytical specificity was defined as the ability of the primers to amplify only the genes of interest in CT and NG, according to the expected product.

Sequencing and analysis of nucleotide sequences

A CT-positive sample presenting both *omp* and Cryptic plasmid genes was chosen; in the case of NG, a sample positive for the *opa* gene was selected, and the control strain ATCC 19424 was used for the *porA* pseudogene. PCR products were used for automated two-way Sanger dideoxy sequencing with the forward and reverse primers described in Table S1, using a genetic analyzer (Macrogen Inc., South Korea). Bioinformatics analyses were performed using the SnapGene software (Dotmatics, USA). The consensus sequences of the different NG and CT genes were aligned against sequences available at NCBI using the Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The fragments of *omp (*181/1274*bp), ORF3 (*459/7500*bp), porA (*184/1348*bp), and opa (*562/1107*bp)* were aligned. The sequences of the CT *omp* and Cryptic plasmid ORF3 genes presented 100% identity with respect to consensus sequences reported in the literature. Likewise, for NG, it was observed that the *porA* pseudogene presented an identity percentage of 100%, while for the *opa* gene, the identity percentages were around 90% concerning consensus sequences.

Regarding the knowledge of CT or NG, five questions were generated per infection. Those who answered four or five questions correctly were classified as having good knowledge, those who answered three correctly were classified as having fair understanding, and those who answered between one and two correctly were considered to have bad knowledge. The questions were whether they have heard about CT or NG, whether having had them (CT and/or NG) at any point grants lifetime immunity, and whether CT and/or NG can be resolved with medication.

Table S1. Genes and primers used

Pathogen	Gen	Reference sequence	Primer	Alignment T°	Amplicon size (pb)
Chlamydia trachomatis	*omp*	AF352789.1	F 5' –CTTTGAGTTCTGCTTCCTCC- 3'	57	181
			R 5' –ACACGGTCGAAAACAAAGTC- 3'		
	Cryptic plasmid ORF3	NC_010029.2	F 5' –GTATATTCTGAGGCAGCTTGC-3'	58	459
			R 5' –ATACGAGCCAGCACTCCAA- 3'		
Neisseria gonorrhoeae	*porA pseudogene*	AJ223449.1	F 5' –CTGGAAAGTAATCAGATGAAACC-3'	57	184
			R 5'- TATCACTCGCTCTGCCGA- 3'		
	opa	X52368.1	F 5' – GCACGGTAAGCGATTATTTC-3'	58	562
			R 5'-TGGGTTTTGAAGCGGGTG-3'		

Internal control	*Human hemoglobin subunit beta (HBB)*	NG_059281.1	F 5' – GAAGAGCCAAGGACAGGTAC - 3' R 5' - GGAAAATAGACCAATAGGCAG -3'	56	408

Supplementary references

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