Microbiological confirmation of a focus of active trachoma in Amerindians of Colombia, South America

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Trachoma is endemic in 57 countries where it is estimated that 40.6 million people have the active forms of the disease: trachomatous inflammation, follicular (TF) and/or trachomatous inflammation, intense (TI). Nearly half (48.5%) of the global charge of active trachoma is concentrated in only 5 countries: Ethiopia, India, Nigeria, Sudan, and Guinea. The World Health Organization (WHO) developed the “SAFE” strategy (Surgery, Antibiotics, Facial cleanliness and Environmental improvement) for the control of trachoma and prevention of blindness, which consists in a timely surgery of the cases of trichiasis, prophylaxis and treatment with antibiotics, personal hygiene, face wash and actions to improve the environment and sanitary conditions. This strategy has helped to diminish significantly the rates of active trachoma and blindness; however, a great number of persons with trichiasis and with risk of blindness still persist.

In South America, active trachoma has only been demonstrated in Brazil, although the Pan American Health Organization (PAHO) has suggested to study the presence of the disease in other border countries. More recently was reported clinical evidence of active phases of trachoma in indigenous communities of the Department of...
Molecular microbiology techniques such as the real-time polymerase chain reaction (PCR) for the detection of C. trachomatis, have demonstrated higher specificity and greater sensibility than the bacterial isolation by cell culture and serologic methods for the diagnosis of trachoma.12,13,14 These tests are of great utility for establishing the presence of active forms of the disease and have been previously used.9,10,15 The aim of this study was to confirm the presence of active trachoma in the focus previously reported in Colombia, by means of carrying out a clinical evaluation and application of molecular microbiology tests for the identification of C. trachomatis.

Methods

A cross sectional study of cases of active trachoma was done by clinical criteria in indigenous communities of the city of Mitú, Department of Vaupés, Colombia. Trips were made to the region where screening was performed by clinical evaluation with emphasis in ocular examination in children who are contacts of patients with trachomatous trichiasis (TT), which corresponds to a chronic phase of the disease; patients were from Santa Catalina community, ethnic group Jupdá-makú previously studied (N 00 21’ 53.79100”, W -70 05’ 53.82706”); additionally, a screening was done with indigenous children of the nutritional recovery center of Mitú, who came from other communities previously unexamined: Pacuativa (N 01’ 19’ 07.74733”, W -70 27’ 52.09842”), Piramirí (01 21’ 56.68124”, W -70 33’ 38.87204”) and San Javier Guaracú ( 01 20’ 12.87522”, W -70 32’ 06.18636”) inhabited by natives of the Cueba ethnicity.

Indigenous children with the clinical forms of active trachoma were transported to the San Antonio Hospital of Mitú where clinical evaluation was done by a clinician specialized in ophthalmology. A selection was made of patients who met the diagnostic criteria of active forms of the disease (TF and/or TI) according to the simplified classification system of the WHO, which defines TF as the presence of five or more follicles of at least 0.5 mm in diameter in the central part of the tarsal conjunctiva and TI (intense) as pronounced inflammatory thickening of the tarsal conjunctiva obscuring more than half the normal deep tarsal vessels.6,7 Patients receiving antimicrobial treatment in the two months previous to the sampling were excluded from the study and demographic and clinical data were registered on a questionnaire (Figure 1).

The aim of the study and the procedure to be performed was explained to the patients and their tutors with the help of an interpreter and informed consent was obtained to proceed with the examination and sampling. General recommendations were provided regarding self-care and protection for children and adults and free treatment was given with Azithromycin in a single dose of 20 mg/Kg for children and of 1 g for adults. The Technical Research Committee and the Ethical Committee of the Instituto Nacional de Salud (INS) in Bogota approved this research.

Obtaining, transportation and storage of samples

In each patient with a clinical diagnosis of active trachoma, two smears were taken from the internal side of the upper eyelid in both eyes using the described technique.9 sterile swabs with rayon tips were used which do not inhibit the PCR reaction.18 These were then introduced into a dry sterile tube, refrigerated at 4°C during its transportation and stored at -20°C until processed in the Laboratory of the Microbiology Group of the INS.

DNA isolation

Both ocular smears of each patient were processed as a single sample according to the recommendation in cases of clinical diagnosis of active trachoma.19 Bacterial DNA present in the samples was extracted and purified with the DNeasy Blood & Tissue kit, according to the manufacturer’s instructions.

Real-Time PCR

Detection of C. trachomatis was done with an “in house” real-time PCR with hybridization probes described by Whiteley et al20 which amplifies a fragment of the ompA gene that codifies for the major outer-membrane protein (MOMP) and its specific of C. trachomatis. LightCycler 480 genotyping master kit was used (Roche Diagnostics GmbH, Mannheim, Alemania), in the amplification reaction, applying the conditions described previously. Donor and acceptor probes (mompP y mompP) were labeled with fluorescein and LC610, respectively, and were added 5 µL of the sample to the reaction mixture. It was developed a real-time PCR employing a monoplex format, which was used as internal amplification control to detect samples with PCR inhibitors that may produce false negative results.21,22 This technique was based in the same thermocycling profile of the assay previously described.20 DNA of the lambda bacteriophage (lambda ladder PFG markers, New England Biolabs) purified with the PureLink Quick Gel Extraction Kit (Invitrogen, Löhne, Germany) was used as target, which was added to each sample (5 pg). In the amplification reaction LightCycler 480 Probe Master Kit (Roche Diagnostics GmbH, Mannheim, Germany), primers lambda-F 5’-CCTGGGTTGCAGATTGCAAA-3’, lambda-R 5’-GGGGCCTGAAATAGCCTTT-3’ (0.4 µM of each), and the probe TaqMan 5’-LC670-CAGCATCGGAGCGTTCGCA-BBQ-3’ (0.2 µM for each reaction mix) which were designed with the software Primer3 (http://frodo.wi.mit.edu/primer3), were used. Positive samples for C. trachomatis with the “in house” real-time PCR described, were confirmed with a second molecular technique (commercial test), real-time PCR assay LightMix 480HT C. trachomatis Kit (TIB MOLBIOL GmbH, Berlin, Germany) which has been employed previously23 and that includes an internal amplification control, for the master mix the LightCycler 480 genotyping master kit.
was used. The amplification was done in a monoplex format using a qualitative detection, evaluating the crossover point (Cp) and the melting temperature (Tm) according to the manufacturer’s instructions. In the described real-time PCR techniques the LightCycler 480 II equipment was used (Roche Diagnostics GmbH, Rotkreuz, Switzerland) using adjusted values of this platform for the analyses of the amplification curves or melting peaks of the amplification products. PCR products obtained were confirmed by electrophoresis in agarose gels 2.5% with 0.2 μg/mL of ethidium bromide and observed in a photo capture system (GelDoc XR, Bio-Rad).

**Nucleotide sequencing**

To validate the obtained results, PCR products of the samples in which *C. trachomatis* was detected by both real-time PCR assays were amplified again with a conventional PCR “booster” standardized in the laboratory using the same primers described by Whiley et al. Nucleotide sequencing was performed on both strands from PCR product using the Sanger method in the Laboratory of Molecular Physiology of the INS. Bioinformatics analysis was done with the Chromas v.1.45 software [Technelysium, Queensland, Australia], ClustalW (http://www.genome.jp/tools/clustalw/) and the BLASTn 2.2.19 -Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov) using the data base of GenBank (http://www.ncbi.nlm.nih.gov).

**Quality controls**

In each DNA extraction batch, a negative control was included adding sterile ultrapure water instead of sample, and in each processing batch of the different PCR techniques, a negative reagent control was added, a negative DNA addition control and a positive control of *C. trachomatis* (included in the commercial assay).

**Results**

**Patients**

The study identified 15 cases of active trachoma, patients had 1 to 12 years old, (mean 4.7 years), 60% were female. The participants originated from indigenous communities of Santa Catalina (73.3%), Pacuativa (20%) and Piramiri (6.7%) (Table 1).

**Clinical and microbiological diagnosis**

Intense trachoma type TI was found in 9 (60%) and the follicular TF in 6 (40%) patients (Figure 2), with a major bilateral commitment of these active forms of the disease. Three patients presented trachomatous scarring (TS) and none of them evidenced TT (trichiasis) or corneal opacity. No patients showed signs of ocular allergy or papillae on upper or lower tarsal conjunctiva. Presence of DNA of *C. trachomatis* was detected in 3 of the

### Table 1. Description of demographic, clinical and microbiologic data of confirmed active trachoma cases in the study.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (Years)</th>
<th>Sex</th>
<th>Origina</th>
<th>Ethnicity</th>
<th>Clinical diagnosis</th>
<th>C. trachomatisb</th>
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<tr>
<td>1</td>
<td>1</td>
<td>F</td>
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<td>Jupdá-makú</td>
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<td>+</td>
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<tr>
<td>2</td>
<td>1</td>
<td>F</td>
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<td>Cubea</td>
<td>TI</td>
<td>-</td>
</tr>
<tr>
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<td>Jupdá-makú</td>
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<td>-</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
<td>3</td>
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<td>Jupdá-makú</td>
<td>TI</td>
<td>-</td>
</tr>
<tr>
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<td>3</td>
<td>M</td>
<td>Santa Catalina</td>
<td>Jupdá-makú</td>
<td>TI</td>
<td>+</td>
</tr>
<tr>
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<td>Cubea</td>
<td>TFd</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Jupdá-makú</td>
<td>TF</td>
<td>+</td>
</tr>
<tr>
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<td>Jupdá-makú</td>
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<td>-</td>
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<tr>
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<td>12</td>
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<td>Santa Catalina</td>
<td>Jupdá-makú</td>
<td>TF, TS</td>
<td>TITS</td>
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</table>

aIndigenous community from which the case proceeded.

bIdentification by real time PCR and sequencing.

cTI=trachomatous inflammation, intense.

dTF=trachomatous inflammation, follicular.

eTS=trachomatous scarring.

Figure 2. Tarsal conjunctiva of patients analyzed in the study with the clinical forms of active trachoma. Fig. 1A: Right eye case 4 with diagnosis of TI, Fig. 1B: Left eye case 2 with TF.
cases of active trachoma (Table 1) with the use of the “in house” real-time PCR. These results were confirmed with the real-time PCR LightMix 480HT kit, in both techniques values of amplification curves and melting peaks were the expected for this bacterial species. (Figure 3)

The obtained findings were validated with nucleotide sequencing of the amplified product in case 6, which showed greater intensity in the electrophoresis band. Bioinformatics analysis revealed an identity of 100% with the sequence of the reference strain of *C. trachomatis* available at GenBank, in the genic region that confers specificity in the identification of this species of *Chlamydia* and corresponding to the hybridization site of the acceptor probe momP2: 5’-TTATGATCGACGGAATTCTGTGGGAA-3. These findings reveal the presence of *C. trachomatis* in ocular samples corresponding to 20% of the cases studied which had 1, 3 and 6 years of age, respectively, whose origin was the Santa Catalina community, and in the clinical evaluation TI diagnosis was done in cases 1 and 6, and TF in case 11. (Table 1)

In the quality controls of the assays employed, amplification was not obtained in any of the negative controls that were included in each batch of DNA extraction, or in conventional and real-time PCR assays, positive controls produced the expected results. The lower detection limit of the "in house" real time PCR technique and of the LightMix 480HT Kit was 102 and 101 genome equivalents for each amplification reaction, respectively. Additionally, no PCR inhibitors were detected in the samples studied with none of the PCR assays used.

**Discussion**

The present study confirms the presence of *C. trachomatis* in clinical cases of active trachoma in indigenous of the department of Vaupés, Colombia, through ophthalmological clinical evaluation according to the criteria established by the WHO and the application of three methods of molecular microbiology: two-real time PCR assays and nucleotide sequencing to ascertain the presence of *C. trachomatis* in the ocular samples studied. The results show *C. trachomatis* in patients with clinical diagnosis of active trachoma in the focus clinically informed by Miller et al.

Cases occurred in young children, age group identified as the principal reservoir of the infection and in which, usually, active forms of the disease occur. Two of the patients that were positive by the PCR techniques correspond to TI cases, in which it is most likely to detect the *C. trachomatis* infection and with a high bacterial charge. Similarly, in this study a greater number of cases were detected by clinical diagnosis than by the real-time PCR assay (15 and 3 patients, respectively), this finding has been observed previously in other studies of active trachoma using different techniques of bacterial DNA amplification in areas of low prevalence of the infection. In a study performed in 670 children clinically diagnosed as active trachoma in 27 villages in Guinea and Pakistan, it was found, using real-time PCR, a global frequency of detection of 23% for *C. trachomatis*: 23.2% and 21.2% for the villages of Guinea and Pakistan, respectively. Similar data to those found in the present study with 20% of positivity for the studied group and for the real-time PCR used. It has been suggested that the difference in the frequency of positivity observed between microbiologic and clinical diagnosis is due to the fact that clinical manifestations of the disease are produced by a marked inflammatory immune response, which disappears more slowly than multiple episodes of infection, due to this may have clinical signs of disease and no bacteria detected in the samples. Additionally, in the present study, quality controls of the different molecular assays employed gave the expected results and no PCR inhibitors were detected in the analyzed samples, therefore, the findings of the molecular tests confirm the cases clinically diagnosed as TF and TI.

In conclusion, the study confirms the cases of active trachoma previously informed in indigenous of the department of Vaupés, Colombia, where it is demonstrated the presence of *C. trachomatis* in this active phase of the disease. The study supports the evidence needed for the inclusion of Colombia in the list of the countries with active trachoma, a disease of health importance in the studied populations, located in remote
regions and of extreme poverty conditions, where disease complications may cause a major impact due to the disability and dependence it produces. It is necessary to establish the magnitude of the problem through prevalence studies and detection of risk factors associated with the infection in the region of study and other parts of the country, as well as in the departments of Guainía and Amazonas, which border regions of Brazil, highly prevalent for trachoma.31 It is recommended that the sanitary authorities take relevant and feasible measures, according to the social and cultural reality of the affected communities, in order to adopt and implement control, prevention and elimination strategies in the region, under the guidelines of the SAFE strategy for the development of intervention plans in these communities. The regime of antibiotic treatment massively by itself does not guarantee the elimination of trachoma. It should must be associated with effective measures of facial cleaning and environmental improvements.33 Additionally it should be necessary to expand the search of trachoma to other countries bordering Brazil to determine the true extent of the disease in South America.

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