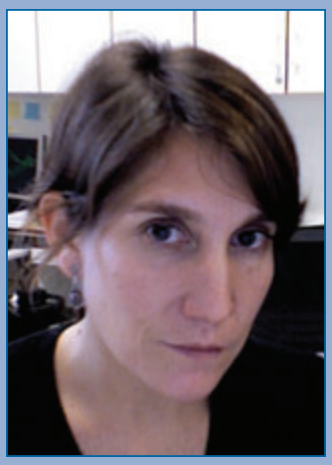

Cristina Guerra-Giraldez

Ms. Guerra-Giraldez studied Biology in her homeland Peru until her MSc degree and then won a fellowship from the German Academic Exchange Office (DAAD) for a PhD on cellular/molecular parasitology in Heidelberg. After some years at Imperial College and the University of York, UK, she returned to her former university, Cayetano Heredia, where she's now a Faculty member at the School of Science and studies brain infection by Taenia solium.

ISID Small Grant Program Report

Adequate Cultures from Clinical Isolates of *Trichomonas vaginalis* for Molecular Studies of Virulence

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Background and Aim

Trichomonas vaginalis is a sexually transmitted eukaryotic extracellular parasite; it adheres to genital epithelia (1). Symptoms are absent in nearly half of the infected women and most of the infected men (2, 3), which suggests differences in aggressivity among isolates. The infection may result in vaginitis, pregnancy complications like low birth weight, cervical neoplasty (4, 5) and increased risk of HIV transmission and replication (6, 7). Trichomoniasis is not diagnosed in public hospitals in Peru because it requires careful culture and sometimes several days of observation. Women with symptoms like itching and odorous discharges, also common in bacterial vaginosis, are treated with metronidazol (8).

Trichomoniasis is easy to cure once detected, but its high worldwide prevalence (174 million new cases in 1999, (9)) and the association with cervical cancer and HIV infection makes it relevant to study the parasite's invasion mechanisms and virulence factors. The complete 160-Mb genome was published (10), raising interest in the genetics and molecular regulation of the parasite in order to explain differences in clinical manifestations, susceptibility to drugs or other variations in pathogenicity (11). Strains (or isolates) have not been classified nor a proper genotyping (12–16) is available.

Our aim is to optimise cultures to free clinical samples of *T. vaginalis* from bacteria and yeast while maintaining populations reliable for molecular assays to study virulence factors and other aspects of the biology of the parasite.

Methods

Clinical samples. 25 women who were being recruited at the Gynecology service at the Hospital Nacional Cayetano Heredia for an unrelated study were chosen by interns as possible positives for *Trichomonas* because of their symptoms. Volunteers signed an informed consent and each provided two vaginal swabs, A and B, applied on the same visit. Swab A was placed on 500 uL of 10mM Tris, 1mMEDTA, pH 7.4 (TE buffer) and swab B on 500 uL of phosphate-buffered saline solution (PBS).

Detection of *Trichomonas*. A 10-uL in-house PCR reaction for a 941-bp fragment of *T. vaginalis* adhesin AP33 was used on the A tubes. Each reaction contained 5.5 uL of sample, 1X Fermentas PCR buffer, 2 mM Mg^{+2}, 5% DMSO, 400 nM forward primer (ACCTCACATTTACAAGAAGAATGC), 400 nM reverse primer (GCCATTCTCTT CATCTCC), 200 nM dNTPs and 1 U of Fermentas *Taq* DNA polymerase (EP0402). The Stratagene Robocycler® 96 Gradient was programmed for 5 min denaturation at 94°C, 40 cycles of 45-sec denaturation at 94°C, 45 sec of hybridation at 53°C and 1 min of elongation at 72°C with a final 5 min at 72°C. Products were run on 1% agarose gels and stained with ethidium bromide; human DNA served as negative control, *T vaginalis* DNA as positive. PCR results were obtained on the same day of sample collection.

Culture start. B tubes were kept at room temperature during the PCR. Positives were spun 3 min at 1500 rpm and the clear liquid was removed. The same tube, with a loose, very small pellet, was filled with 1.35 mL of pre-warmed TYM medium (2% tryptone, 1% yeast extract, 1.25% maltose, 0,1% L-cysteine, 0,02% ascorbic acid, 17,25mM K_2HPO_4 and 17,5 mM KH_2PO_4, pH 6,2), 150 uL of heat-inactivated (56°C for 30min) filtered human serum (from a pool of 6 healthy donors) and low doses of antibiotics (62,5 ng/ul Gentamycin, 210 ng/ul Penicillin, 450 ng/ul Streptomycin and 2,5 ug/ul Amphotericin B), for overnight incubation at 37°C. Afterwards 4.5 mL of the same TYM + serum + antibiotics were added to the 1.5-mL starting cultures in appropriate glass tubes, and incubated at 37°C in an inclinated position (a 40–50° angle). Counts at 100X and 400X were done every second day.

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Axenization. We used an axenisation method kindly shared by Dr. Rossana Arroyo, from the Infectomics and Molecular Pathogenesis lab at the "Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional" (CINVESTAV-IPN), Mexico. Briefly, cultures were washed three times with pre-warmed PBS and media were replaced doubling the concentration of antibiotics; two different dilutions from each contaminated culture were made, and two similar ones were cultured and diluted or passaged, without antibiotics. We added 10 ug/mL ciprofloxacin and 30 ug/mL chloramphenicol. Cultures were counted every other day.

Results

Five of the 25 A swabs were PCR positive for *Trichomonas*; the corresponding B swabs were cultivated. All showed motile parasites between days 3 and 7, but two of them did not grow enough to allow for passages. The other three showed different degrees of contamination with bacteria; one of them also with yeast. All three were successfully cleaned within one or two weeks using Arroyo's procedure.

Discussion

The association between clinical manifestations, genotype and genetic regulation in *Trichomonas vaginalis* needs reliable molecular studies and axenic cultures from infected subjects instead of laboratory isolates. Axenization of clinical isolates was possible using a protocol established in Mexico (R. Arroyo, CINVESTAV-IPN). We used human serum in the TYM medium instead of horse serum; cryopreservation with this supplement remains to be tested.

The gold standard for the detection of *T. vaginalis* is microscopic observation, but it takes long time; PCR resulted more practical. The in-house reaction was based on a protein exclusive to *Trichomonas*, adhesin AP33; the primers had been tested for cross-reaction with *Neisseria*, *Chlamydia*, *Candida* and human DNA.

Most of the women in the small group of volunteers (20/25) were recruited because of their symptoms but turned to be PCR negative to *Trichomonas*. As the rule is symptomatic management, these women could have needlessly received metronidazole. On the other hand, due to the high frequency of asymptomatic infections (2), many infected people rutinarily escape the medical scrutiny.

Besides studies at the expression level, such as the iron-inducibility of virulence factors (17), molecular epidemiology of strains present in different communities or geographical areas should provide valuable information about this infection. The obtention of axenic clinical samples using human serum and serial passages with different concentrations of antibiotics is simple and relatively unexpensive, and together with complete clinical histories should enable valuable future studies for focused prevention and treatment.

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