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***Lophomonas blattarum* DETECTION IN METHYLENE BLUE-STAINED SPUTUM SAMPLES FROM ADULTS INDIVIDUALS INHABITANTS OF AN URBAN AREA OF CHIRIQUI PROVINCE**

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ABSTRACT

Lophomonas is a genus of multi-flagellated protozoans consisting in two species *Lophomonas striata* and *Lophomonas blattarum*. Both species are considered endocommensal parasites of some domestic pest insects. While *L. striata* has no importance in public health, *L. blattarum* is the causal agent of lophomoniasis, a respiratory tract parasitic infection that mostly affect children and immunocompromised adults. A previous study from our group, reported an important prevalence of *L. blattarum* in the intestinal tract of American cockroaches (*Periplaneta americana*), collected in the peridomiciliary zone of houses from an urban area of Panamá. Based on this, we aimed to screen by Methylene Blue-staining the sputum samples from immunocompetent adults (N=80) with bronchopulmonary affections to

detect potential carriers of *L. blattarum*. A total of 53 individuals were included in the analysis. We observed the presence of *L. blattarum* in 19 out of 53 wet Methylene blue-stained samples. Our results point out a substantial prevalence (35.8%) of *L. blattarum* positive individuals in an area with high incidence of respiratory problems. In addition, we propose the use of Methylene blue stain as a quick and economic diagnostic tool; this reagent highlights the morphological structures of the parasite making easy its identification.

KEY WORDS

Sputum, lofomoniasis, Methylene blue-stain, identification, diagnosis.

DETECCIÓN DE *Lophomonas blattarum* EN MUESTRAS DE ESPUTO TEÑIDAS CON AZUL DE METILENO DE INDIVIDUOS ADULTOS DE UN ÁREA URBANA DE LA PROVINCIA DE CHIRIQUÍ.

RESUMEN

Lophomonas es un género de protozoos flagelados que contiene dos especies, *Lophomonas striata* y *L. blattarum*. Ambas son consideradas endocomensales parásitos de insectos domésticos. Aunque *L. striata* no se considera importante en salud pública, *L. blattarum* es el agente causal de una infección respiratoria llamada lofomoniasis que afecta a niños y a adultos inmunosuprimidos. Un estudio previo, reportó importante prevalencia de *L. blattarum* en el tracto intestinal de especímenes de *Periplaneta americana* colectados en el peridomicilio de zonas urbanas de la República de Panamá. Analizamos mediante tinción con azul de metileno muestras de esputos de adultos que presentaban afecciones broncorespiratorias con el objetivo de detectar la presencia de *L. blattarum* en potenciales portadores. Se analizaron 53 muestras y se observó la presencia de *L. blattarum* en 19 muestras teñidas con azul de metileno. Nuestros hallazgos señalan una importante prevalencia (35.8%) de *L. blattarum* en muestras de esputo de individuos que viven en una zona con alta incidencia de problemas respiratorios. Estos resultados basados en tinción con azul de metileno, destacan la utilización de esta técnica rápida y económica como metodología diagnóstica. Este reactivo resalta las estructuras morfológicas del parásito haciendo fácil su identificación y diagnóstico en muestras de esputo.

PALABRAS CLAVE:

Espudo, lofomoniasis, azul de metileno, identificación, diagnóstico.

INTRODUCCIÓN

Lophomonas blattarum, is a multi-flagellated protozoan parasite (Ruggiero *et al.*, 2015), and the causal agent of pulmonary lophomoniasis, a respiratory tract parasitic infection that can affect either immunocompromised or immunocompetent individuals (Saldana *et al.*, 2017, Tyagi *et al.*, 2016). As an endocommensal parasite, the protozoan inhabit the digestive tract of some household pest insects such as the American cockroaches (*Periplaneta Americana* L) (Sobarzo *et al.*, 2015, Strand and Brooks, 1977), and the German cockroaches (*Blattella germanica* L) (Martinez-Giron *et al.*, 2017). These cockroaches are regarded as vectors for this multi-flagellated protozoan because the parasite is eliminated through the feces and form protective cyst that survive in difficult conditions (Kudo, 1931, Martinez-Giron and van Woerden, 2014). The viable cyst that remain in the environment, could be inhaled or ingested by the human host infecting lung, the maxillary sinuses or other organs including the genitourinary system (Xue *et al.*, 2014, He *et al.*, 2011, Martinez-Giron and van Woerden, 2014). Base on this, lophomoniasis can be considered as a vector-borne zoonotic infection. To prevent *L. blattarum* infection is necessary to control the source of infection including insect vectors such as the cockroaches and termites (Xue *et al.*, 2014).

In the past two decades few cases have been reported in the medical literature reviewed in (Xue *et al.*, 2014) including 136 cases in China, 6 cases in Peru and very few cases in Spain, the United Arab Emirates and India, suggesting that *L. blattarum* infections are rare or have been misidentified or misdiagnosed at hospital level.

The clinical presentation of bronchopulmonary lophomoniasis includes a broad spectrum of nonspecific symptoms, ranging from mild cough to severe respiratory insufficiency with purulent exudates, high fever and radiological signs of pulmonary consolidation (Zhang *et al.*, 2011, Saldana *et al.*, 2017, Martinez-Giron and van Woerden, 2014).

L. blattarum infections have been reported as coinfection with *Mycobacterium tuberculosis* (Verma *et al.*, 2015) or with fungi. The fact

that lophomoniasis is difficult to differentiate from other common infections with similar symptoms (such as bacterial pneumonia or bronchitis) due the nonspecific clinical manifestations and laboratory tests (Xue *et al.*, 2014) make difficult the accurate diagnosis.

The use of drugs for other common non-parasitic respiratory tract infections were almost useless for *L. blattarum* infection (Xue *et al.*, 2014). However metronidazole and/or tinidazole have been used for the treatment of this pulmonary infestation in a number of case reports with satisfactory outcomes (Martinez-Giron and van Woerden, 2014, Xue *et al.*, 2014).

In the reported cases (Xue *et al.*, 2014), the identification of *L. blattarum* in human samples was based on the morphological features of the protozoon observed in respiratory secretions under optical microscopy (Martinez-Giron and van Woerden, 2013). *L. blattarum* was detected in fresh and stained samples of the respiratory tract including sputum, broncho-alveolar lavage, bronchoscopy biopsy smears, bronchial brushing, and tracheal aspirates (Xue *et al.*, 2014). Staining methods for *L. blattarum* diagnosis include mostly Mallory's trichromic stain or a combination of Papanicolaou and Giemsa stains or Wright-Giemsa stains (Martinez-Giron and van Woerden, 2014, Xue *et al.*, 2014).

In a previous study we observed under optical microscope trophozoites or cyst of *L. blattarum* and we reported a prevalence of *L. blattarum* (8.16%) in 49 intestinal tract samples of *P. americana* collected in an urban area of Panamá (Sobarzo *et al.*, 2015).

To offer new insight into the epidemiology of lophomoniasis in human samples from Panama, the purpose of this study was to estimate the prevalence of *L. blattarum* by analyzing Methylene blue-stained sputum samples obtained from patient with respiratory symptoms. We observed the presence of *L. blattarum* in 19 out of 53 individuals from an area with high incidence of respiratory problems.

METHODS

Study design and patient population

We carried out a qualitative, descriptive, cross-sectional study applying simple non-probabilistic randomized sampling. Sample processing and analysis was conducted at the Department of Microbiology and Parasitology, School of Biology, Faculty of Natural and Exact Sciences of the Universidad Autónoma de Chiriquí (UNACHI). Between January and June 2017, we recruited as voluntary anonymized participants, 80 adult of both sexes presenting bronchopulmonary symptoms and recurrent respiratory conditions accompanied by nasal secretion and/or persistent cough with mucopurulent expectoration.

Sample processing and analysis

Individuals were instructed to collect the first expectoration of the morning in a screw-capped 50 mL plastic container. Samples were transferred to the Laboratory L-15 at the School of Biology (UNACHI) and processed following the biosafety standards protocols. Briefly, under an EdgeGARD laminar flow hood (EG3252), a sterile wooden tongue depressor was used to homogenize and prepare three wet samples: 1) direct unstained smear, 2) iodine parasitological solution stained smear and 3) Methylene blue stained smear. Each preparation was mounted with a coverslip and observed under a Leica zoom 2000 optical light microscope at a magnification of 10x and 40x. *L. blattarum* trophozoites and cysts forms were described and identified based on the morphological characteristics of the genus observed under optical microscopy. Images were obtained with a photo camera Canon and saved in folders to post hoc discussion with the team. Identification and descriptive criteria were based on Singh *et al.*, 2013 (Singh and Madan, 2015) and Martinez-Girón *et al.* (Martinez-Giron and van Woerden, 2013) as follow: trophozoite has an ovoid or spherical shape (Singh and Madan, 2015), a round-oval nucleus at the basal end of the cell, a plume of anterior flagella. Fully formed cysts are spherical or oval surrounded by a homogeneous membrane (Martinez-Giron and van Woerden, 2013). Both trophozoites and cysts were identified in the samples.

RESULTS

According to the inclusion criteria, 80 individuals were recruited between January and June 2017 in a qualitative, descriptive and cross-sectional study, nevertheless 53 samples were included in the final analysis. Three wet samples were prepared as 1) direct unstained smear, 2) iodine parasitological solution stained smear and 3) Methylene blue stained smear and all of them observed under light optical microscope. According to morphological observations *L. blattarum* was found in 19 out of 53 fresh and stained sputum samples accounting for a total prevalence of 35.8%. Trophozoites were observed in 11 out of 53 samples (21% prevalence) and cysts were observed in 8 out of 53 samples (15% prevalence).

The Methylene blue staining allowed us to differentiate the structures present in the multiflagellate parasite (Figure 1). At the cyst stage we observe the nucleus as a bluish structure surrounded by the light blue stained cytoplasm (Fig. 1A). At the trophozoite stage we can observe different structures such as the nucleus as an intense blue structure and the slightly semitransparent blue plume of flagella (Fig. 1B). The double membrane is highlighted as a semitransparent structure (Fig. 1C), and the light blue granular cytoplasm containing the semitransparent delimited vacuoles and nucleus (Fig. 1D). With this staining methodology we could identify clearly the kinocilium of hair cells (Fig. 1E), that can be confused with a protozoan such as *Trichomonas*, *Lophomonas* and *Balantidium* (Martinez-Giron and van Woerden, 2013). The results of the direct wet sample and wet iodine staining were not included in the study.

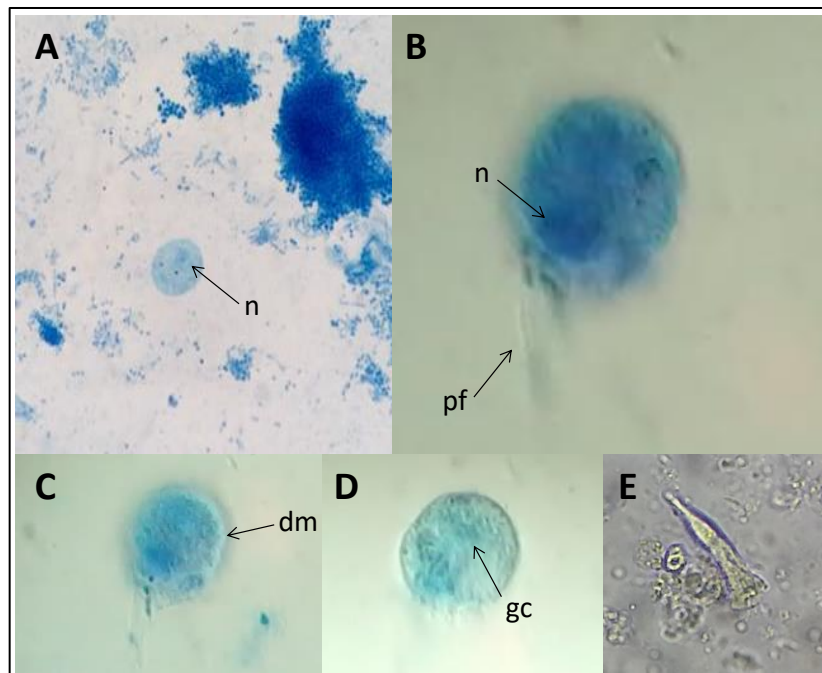


Fig. 1 Methylene blue-stained sputum sample showing the morphological structures. A *L. blattarum* cyst (100X), blue-stained nucleus is highlighted; B *L. blattarum* trophozoite (100X), the plume of flagella (pf) and the nucleus (n) are observed; C *L. blattarum* trophozoite (40X), showing double membrane (dm); D *L. blattarum* trophozoite (40X) showing the granular cytoplasm (gc) containing vacuoles and nucleus. E Ciliated cell (40X).

DISCUSSION

L. blattarum has been recognized as the causative agent of a respiratory tract infections, called lophomoniasis which may affect either immunocompromised or immunocompetent individuals. *Lophomonas blattarum* is a multi-flagellated endocommensal protozoan parasite that inhabits the hindgut of some insects including the American cockroaches *Periplaneta americana*. This specie of cockroach is worldwide distributed, present in tropical climates and commonly

associated with human activities. The cyst forms of *L. blattarum* could be discharged with the secretion and excrements of its vectors. In this sense the cockroaches may act as mechanical vectors for *L. blattarum*, spreading the infective viable cyst forms by contaminated dust or food easily infecting the human host.

L. blattarum has been considered as an opportunistic pathogen in individuals with suppressed immunity. However in 2016 was reported a case of lophomoniasis in an immunocompetent 23 years old male, which suggest the ability of the parasite to infect either immunocompromised or immunocompetent patients. In a previous study we report an important prevalence of *L. blattarum* (8.16%) in the intestinal tract of *P. americana* collected in the peridomiciliary zone of houses in an urban area of Chiriquí province, Republic of Panamá (Sobarzo *et al.*, 2015). The presence of cockroaches such as the *Blattella germanica* in the environment is a recognized risk factor for asthma (Sohn and Kim, 2012, Do *et al.*, 2016) we suggested that the presence of *P. americana* could be linked as a risk factor of respiratory affections, as these insect could serve as vector for many pathogens organisms. The mechanism of transmission for *L. blattarum* has not been clearly described, and the vector borne is the only transmission pathway proposed so far. However person-to-person transmission should also be considered precisely because vertical transmission could happened and thought the presence of cyst in sputum samples.

Based on this we proposed to screen immunocompetent adults (18-36) presenting bronchopulmonary affections accompanied by nasal secretion and/or persistent cough with mucopurulent expectoration to detect potential carriers of *L. blattarum*. We observed the presence of *L. blattarum* in 19 out of 53 individuals from an area with high incidence of respiratory problems. Common cold, seasonal flu or asthma are the more frequent causes of bronchopulmonary affections. However the results we present in this study suggest that lophomoniasis could account as a potential cause of respiratory conditions that have not been diagnosed at hospital level. This is the first report of *Lophomonas blattarum* in sputum samples from patients with respiratory symptoms from an urban area of the Republic of Panama. We suggest that the

presence of *L. blattarum* in sputum samples is associated with these symptomatology. We considered this is an important and high relevant study because patients recruited as anonymized volunteers participants, presented recurrent bronchopulmonary problems with no response to treatment.

Immunocompromised individuals are very often affected by pulmonary affections and treated with the medication for the common infections (e.g. seasonal flu or TB), not considering lophomoniasis as a potential cause of respiratory complication. The wrong diagnosis and incorrect drug treatment at hospital level could lead to unnecessary hospitalization. This is an obvious importance for clinicians to consider *Lophomonas blattarum* in their differential diagnosis as a possible causative agent of respiratory symptoms.

Our results point out the use of Methylene blue staining as a quick and economic diagnostic tool for the detection of *Lophomonas* spp at diagnostic laboratory. The results of the direct wet sample and wet iodine staining were discarded for poor quality in the observation of parasite structures. However, Methylene blue reagent highlights the morphological structures of the parasite making easy its identification (Figure 1). This staining method is simple, fast and economical and allowed us to observe the different structures of the trophozoite and cysts. Therefore, we recommend to implement the routine use of this method as a quick and easy diagnosis tool in the identification of *L. blattarum* in fresh bronchopulmonary samples.

AUTHOR CONTRIBUTIONS

Conceived the study: RS, MP, GV, AHM-T.

Processed the samples: RS, MP, GV.

Analyzed the data: RS, MP, GV, AHM-T.

Contributed reagents/materials/analysis tools: RS, MP, GV, AHM-T.

Wrote the paper: RS, MP, GV, AHM-T.

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COMPETING INTERESTS:

The authors have declared that no competing interests exist.

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