Parasitol Res (2015) 114:3309–3314
DOI 10.1007/s00436-015-4554-4

ORIGINAL PAPER

Identification criteria of the rare multi-flagellate *Lophomonas blattarum*: comparison of different staining techniques

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Received: 9 May 2015 / Accepted: 25 May 2015 / Published online: 3 June 2015

Abstract Bronchopulmonary lophomoniasis (BPL) is an emerging disease of potential importance. BPL is presented by non-specific clinical picture and is usually accompanied by immunosuppression. Culture of *Lophomonas blattarum* is difficult and its molecular diagnosis has not yet been developed. Therefore, microscopic examination of respiratory samples, e.g., bronchoalveolar lavage (BAL) or sputum, is the mainstay of BPL diagnosis. Creola bodies and ciliocytophthoria are two forms of bronchial cells which occur in chest diseases with non-specific clinical picture like that of BPL. Both forms could be misrecognized as multi-flagellates because of their motile cilia in the wet mounts and due to shape variability of *L. blattarum* in stained smears. The aim of the study is to compare different staining techniques for visualizing *L. blattarum* to improve the recognition and diagnosis of BPL, to distinguish respiratory epithelial cells from *L. blattarum* and to decide which stain is recommended in suspected cases of BPL. BAL samples from patients which contain *L. blattarum*, creola bodies, and ciliocytophthoria were collected then wet mounts were examined. The BAL samples were also stained by Papanicolaou (PAP), Giemsa, hematoxylin and eosin (H & E), trichrome, Gram, and Diff-Quik (DQ) stains. The different staining techniques were compared regarding the stain quality. In wet mounts, the ciliary movement was coordinate and synchronous while the flagellar movement was wavy and leaded to active swimming of *L. blattarum*. In stained slides, bronchial cells were characterized by the presence of basal nucleus and the terminal bar from which the cilia arise. Trichrome was the best stain in demonstration of cellular details of *L. blattarum*. H & E, PAP, and Giemsa stains showed good quality of stains. Gram and DQ stains showed only pale hues of *L. blattarum*. We recommended adding Wheatley's trichrome staining to the differential diagnosis workup of cases of non-specific chest infections, especially when BPL is suspected, to avoid overdiagnosis or underdiagnosis of it.

Keywords *Lophomonas blattarum* · Lophomoniasis · Trichrome · Ciliocytophthoria · Creola bodies · PAP · Bronchoalveolar lavage (BAL) · Giemsa

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Introduction

Lophomonas blattarum belongs to *Lophomonas* of Lophomonadae in Hypermastigida and Lophomonadina of Mastigophora in protozoa (Farmer 1980). *L. blattarum* is a multi-flagellated parasite which inhabits the hindgut of termites and Dictyopteris (cockroaches) as an endocommensal (Strand and Brooks 1977). It is subsequently eliminated from the hindgut in feces and it can encyst in adverse external environment conditions, as has been shown for other flagellated protozoa (Chavez-Munguya et al. 2007; Bittencourt-Silvestre et al. 2010; Zaragatzki et al. 2010). *L. blattarum* can pollute numerous things, including dust through the crawling of termites and cockroaches. *L. blattarum* can parasitize the lung of the patient through inhalation of dust (Wang et al. 2006). The existence of various species of protozoa, pathogenic and nonpathogenic, was reported in the hindgut of cockroaches (Brugerolle et al. 2003; Pai et al. 2003).

Bronchopulmonary lophomoniasis (BPL) is an emerging disease of potential importance (Yao 2008; Wu and Liu 2010; Zhang et al. 2011). About 70 cases were identified in the literature. The majority of these reports were from China, with some cases from Peru and Spain. Immunosuppression was a feature in a number of the case reports. Clinical presentation was non-specific, including symptoms such as fever, cough, and breathlessness. All the cases in the reviewed literature had evidence of previous and/or concomitant respiratory disease. These cases showed pulmonary infiltrate in chest computed tomography (CT). Around 35 % of patients had eosinophilia. Antiprotozoal therapy was generally effective (Martinez-Giron and Doganci 2010; Zerpa et al. 2010; Martinez-Giron 2013; Martinez-Giron and van Woerden 2013).

Lophomonas blattarum is difficult to culture than many other protozoa living in the gut of cockroaches. However, it has been grown in three mediums utilized by Chen (1933) and in a medium which contained 0.8 % salt solution with yeast added as food (Kirby 1950). Moreover, no molecular characterization of *L. blattarum* was developed. Therefore, the identification of this multi-flagellate in human samples has been based on the identification of morphological features under light microscopy using fresh and stained samples from the airways including sputum, bronchoalveolar lavages (BAL), bronchial brushings, and tracheal aspirates (Ribas et al. 2007; Martinez-Giron et al. 2011).

Multi-flagellate protozoa are difficult to differentiate from ciliated bronchial epithelial cells, and misidentification under light microscopy is a significant risk. In fresh samples, the motile cilia of the ciliated respiratory epithelial cells could be easily misidentified as flagellated protozoa. In stained smears, the creola bodies (small groups of ciliated bronchial cells) and ciliocytophthoria (detached ciliary tufts with cytoplasmic remnants) are misrecognized as multi-flagellates (Ribas et al. 2007; Martinez-Giron et al. 2011; Martinez-Giron and van Woerden 2014).

BAL samples sent to laboratory are most probably subjected to Papanicolaou (PAP), Giemsa, hematoxylin and eosin (H & E), Gram, Wheatley trichrome and Diff-Quik (DQ) staining. Therefore, the aim of our study is to compare different staining techniques for visualizing *L. blattarum* to improve the recognition and diagnosis of BPL, to distinguish respiratory epithelial cells from *L. blattarum*, and to decide which stain is recommended in suspected cases of *L. blattarum* infection.

Materials and methods

Parasite material and design of the study

Parasite material was collected from BAL samples, of a patient with an end-stage renal disease who complained of fever, severe cough, and expectoration. The blood picture showed eosinophilia and the CT scan showed severe lung infiltration. The case was not improved with regular antibiotic treatment and showed complete rapid improvement on metronidazol.

BAL samples that contain creola bodies and ciliocytophthoria were also collected. Informed consents were taken from the patients. Samples were subjected to wet mount examination and different staining techniques.

Staining procedures

Cytospins and smears were prepared, allowed to dry, and stained by the following stains:

1. Papanicolaou (PAP) stain (Koss 1992):
   Immediately fixed in 95 % ethanol, rinse in water (10 dips), Gill's hematoxylin for 1 min, rinse in water until water is clear, 0.5 % ammonia water for 1 min, rinse in water, modified orange G for 1 min, two changes of 95 % ethanol (10 dips each), EA 50 for 1 min, two changes of 95 % ethanol (10 dips each), two changes of 100 % ethanol 2 min each, two changes of xylene 2 min each, and two changes of xylene 5 min each.
2. Giemsa stain (El-Sayed and Hikal 2014):
   Absolute methanol for 30 s (for fixation) and 20 % fresh Giemsa solution in pH 7.2 of phosphate buffer for 20 min.

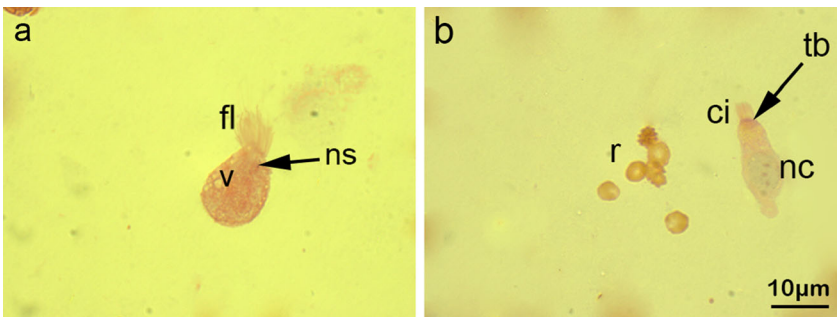

Fig. 1 PAP-stained slides. **a** *L. blattarum*; irregular long flagella (*fl*) arise from the anterior part. Nucleus is not well apparent, only nuclear shadow (*ns*) appears. Some cytoplasmic vacuoles (*v*) are visible. **b** Bronchial cell; regular short cilia (*ci*) arise from the terminal bar (*tb*) at the luminal border of the cell. The nucleus (*nc*) is at the base of the cell. Some RBCs (*r*) appear in the field. (×1000)

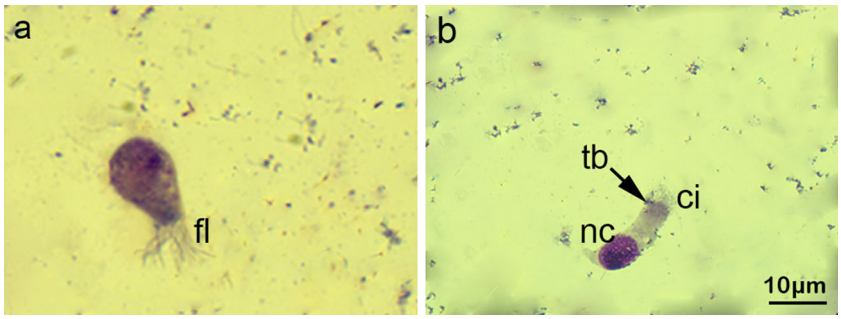

Fig. 2 Giemsa-stained slides. **a** *L. blattarum*; irregular long flagella (*fl*) arise from the anterior part. The nucleus is not apparent. **b** Bronchial cell; short cilia (*ci*) arise from the terminal bar (*tb*) at the luminal border of the cell. The nucleus (*nc*) is at the base of the cell. (×1000)

3. Hematoxylin and eosin (H & E) stain (Kiernan 2008):

   Two changes of xylene for 10 min each, two changes of absolute ethanol for 5 min each, 95 % ethanol for 2 min, 70 % ethanol for 2 min, rinse in water, stain in hematoxylin solution for 8 min, rinse in water for 5 min, 1 % acid alcohol for 30 %, rinse water for 1 min, 0.2 % ammonia water for 1 min, rinse in water for 5 min, 95 % ethanol (10 dips), eosin for 1 min, 95 % ethanol for 5 min, two changes of absolute ethanol for 5 min each, and two changes of xylene for 5 min each.
4. Wheatley's trichrome stain (Garcia 2007):

   Schaudinn's fixative for 30 min, 70 % ethanol for 5 min, 70 % iodine alcohol (70 %) ethanol for 1 min, two changes of 70 % ethanol for 5 min, trichrome stain for 10 min, acetic acid alcohol (90 % ethanol) for 1 to 3 s, dipping in 100 % ethanol, two changes of 100 % ethanol for 3 min each, and two changes of xylene for 5 min each.
5. Gram stain (York 2004):

   Heat fixation, crystal violet for 1 min, rise in water, Gram's iodine for 1 min, rinse in water, 95 % ethanol for 30 s, rinse in water, 0.25 % safranine for 1 min, and rinse in water.
6. Diff-Quik stain (DQ, modified Giemsa stain) (Skipper and DeStephano 1989):

   Air-dried slides were fixed in Diff-Quik fixative (1.8 mg/L triarylmethane in methyl alcohol) for 30 s, rinse in water, Diff-Quik solution 2: made of thiazine dye mixture: 1.25 g/L of pure dye (0.625 g/L Azure A and 0.625 g/L methylene blue) and phosphate buffer (pH 6.6) for 30 s, Diff-Quik solution 1: made of xanthene dye, 1 g/L of pure

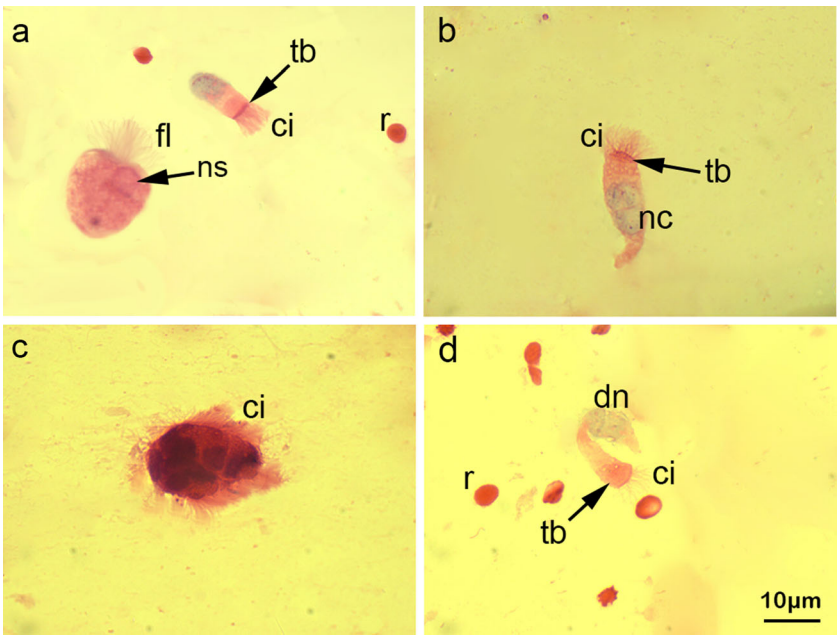

Fig. 3 H & E-stained slides. **a** *L. blattarum*; irregular long flagella (*fl*) arise from anterior part. Nucleus is not well apparent, only nuclear shadow (*ns*) appears. Some RBCs (*r*) appear in the field. **b** Bronchial cell; regular short cilia (*ci*) arise from the terminal bar (*tb*) at the luminal border of the cell. The nucleus (*nc*) is at the base of the cell. **c** Creola body; cluster of detached bronchial cells which are ciliates (*ci*). **d** Ciliocytophthoria; degenerated bronchial cell in which a pinching off occurs between the cytoplasm bearing the cilia (*ci*) which arise from the terminal part, and the nucleated cytoplasm, resulting in a mass of cytoplasm-bearing cilia without a nucleus, and a degenerating nucleus (*dn*) with cytoplasm. In some degenerated bronchial cells, the cilia-bearing cytoplasm was completely separated from the degenerating nucleus. Some RBCs (*r*) appear in the field. (×1000)

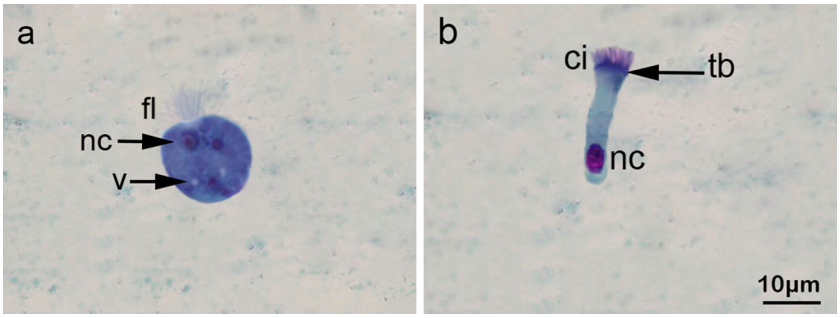

Fig. 4 Trichrome-stained slides. **a** *L. blattarum*; irregular long flagella (*fl*) arise over the anterior part which are longer at center and shorter at sides. The vesicular nucleus (*nc*) is well apparent. Some cytoplasmic vacuoles (*v*) are visible among the granular cytoplasm. **b** Bronchial cell; regular short cilia (*ci*) arise from the terminal bar (*tb*) at the luminal border of the cell. The nucleus (*nc*) is at the base of the cell. (×1000)

dye, phosphate buffer (pH 6.6), and sodium azide (0.01 %) as preservative for 30 s, and rinse in water.

Identification criteria and evaluation of different staining techniques

All stained slides, as well as wet mounts of BAL samples, were examined with Leica DM 2500 microscope at a magnification of ×400 and ×1000. Photographs were obtained using a Leica DFC 500 digital camera.

Different staining techniques were compared regarding the stain quality and the ability to demonstrate the flagella and the cellular content. A set of morphological criteria to differentiate *L. blattarum* from ciliated cell fragments in wet mount and stained slides were also described.

Results

All results are shown in Figs. 1, 2, 3, 4, and 5; Tables 1 and 2; and videos 1 and 2.

Discussion

Clinical picture of BPL is not specific, and therefore should be differentially diagnosed from other chest diseases. BPL should be suspected in cases of immunosuppression with severe chest infection, eosinophilia, and failure of response to regular antibiotic treatment.

Two morphological forms of respiratory epithelial cells, creola bodies and ciliocytophthoria, could be mistaken for *L. blattarum*. Creola bodies are clusters of desquamated epithelial cells that were reported in cases of bronchial allergy and infections (Yoshihara et al. 2006; Yamada and Yoshihara 2010). Ciliocytophthoria (or detached ciliary tufts) is a peculiar degeneration of the ciliated respiratory epithelium in which a pinching off occurs between the cilia-bearing cytoplasm and the nucleated cytoplasm, resulting in an anucleated mass of cytoplasm-bearing cilia and a degenerating nucleus with cytoplasm which are called ciliocytophthoria (Johnston and Elson 2008).

In wet mounts of our study, bronchial cells, ciliocytophthoria and creola bodies showed motility of cilia and therefore could be misdiagnosed as *L. blattarum*. Shakoor et al. (2011) and Khan et al. (2015) reported the presence of motile ciliary tufts that can mimic the motility of flagellated amoebae in wet mounts of CSF and presents a diagnostic dilemma. Kuritzkes et al. (1988) reported that these detached ciliary tufts retain the mitochondria and therefore remain motile for days.

Wet preparation has no permanent record unlike the permanent stained smears which can be used for consultations with specialists (Clavel et al. 1999; Garcia 2007). Moreover, morphological details are more readily seen by permanent stained smears (Aykan et al. 2005).

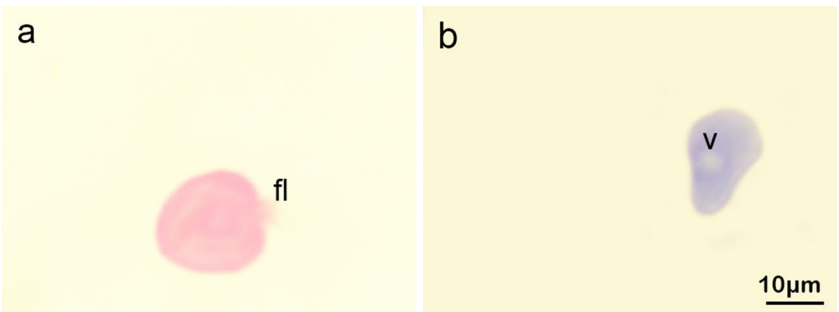

Fig. 5 **a** Gram-stained slide. Pinkish hue of *L. blattarum* with remanent flagella (*fl*). **b** DQ-stained slide. Violet hue of *L. blattarum* with poorly visible vacuole (*v*) (×1000)

Table 1 Comparison of different staining techniques for visualizing *L. blattarum*

Stain	Color of *L. blattarum*	Flagella appearance	Comment(s) about staining quality
Papanicolaou	Orange to pink	Faint but sharply defined	Good quality
Giemsa	Purple	Strongly stained but hazy	Good quality
Hematoxylin and eosin	Pink	Well-stained and sharply defined	Very good quality
Trichrome	Bluish green with purple tinge	Strongly stained and sharply defined	Best quality
			Details are well stained
Gram stain	Pink (Gram positive)	Not stained	Poor quality, only pinkish hue
			No details, appears like remnants or ghost of the parasite
Diff-Quik stain	Purple	Not stained	Poor quality, only violet hue
			No details, appears like remnants or ghost of the parasite

In stained slides of our study, all forms of bronchial cells could be differentiated from *L. blattarum* by the presence of the basal nucleus and/or terminal bar. Similar findings were reported by Martinez-Giron and van Woerden 2013.

In the present study, *L. blattarum* was reported to have different shapes varying from oval, rounded, to pyriform. This may be due to its plasticity or because it has different life stages forms (Kudo 1954; Brugerolle and Lee 2000). The shape variability of *L. blattarum* leads to more confusion in its identification in the stained smears. We found that the nucleus of *L. blattarum* is not usually visible. Similar observation was reported by Brugerolle and Lee (2000). Kessel and Beams (1990) reported that the nucleus of *L. blattarum* is usually hidden inside a funnel-shaped space, which is formed by axial filaments.

In the present work, the trichrome stain was the best to show morphological details of *L. blattarum* followed by the H & E stain. Trichrome stain gave uniform results and is effective in confirming the diagnosis of BPL. Satisfactory results were also obtained with PAP stain and Giemsa stain; however, they were of medium quality. *L. blattarum* were stained irregularly with Gram and DQ stains. Most of them showed only a slight pinkish or violet hue by Gram or DQ stain, respectively.

Deficiency of awareness of the presence of creola bodies and ciliocytophthoria will increase the risk of overdiagnosis of

Table 2 Morphological criteria for differentiation between *L. blattarum*, bronchial cells, creola body, and ciliocytophthoria

Characteristic	*L. blattarum*	Bronchial cells	Creola body	Ciliocytophthoria
Size	20–35 μm	up to 20 μm	Variable	Variable
Shape	Plastic: pyriform, rounded, or oval	Columnar	Clumps or rounded clusters of bronchial cells which are characterized by the presence of cilia all around the bodies or on the top of the cluster of the detached epithelial cells (video 2)	Triangular part of the bronchial cells bearing cilia, which is separated from the cytoplasm bearing the degenerating nucleus
Cytoplasm	Cytoplasmic granules and vacuoles	Clear		
Nucleus	Vesicular Usually not seen	Basally located nucleus that has fine granular chromatin	Like bronchial cells	Degenerating nucleus in a separated part of the cytoplasm
Flagella/cilia	Long Undulating	Short Straight	Like bronchial cells	Like bronchial cells
	Variable in length	Uniformed length		
	No terminal bar	Thick terminal bar		
	At one end of the cell	Inserted in the terminal bar at the luminal border of the cell		
	Wavy movement which leads to active swimming of the parasite in wet mount More rapid (video 1)	Coordinate and synchronous movement which does not lead to active movement of the cells in wet mount (video 2)		

BPL especially when the clinical picture strongly indicates BPL. Moreover, usually, BAL samples sent to the cytology laboratory, are subjected to PAP or Giemsa staining. Both stains showed medium quality in identification of *L. blattarum*, which may lead to misidentification of *L. blattarum* by insufficiently experienced personnel. On the other hand, BAL samples sent to microbiological laboratories are usually stained by Gram stain which showed pink hues of *L. blattarum*. Therefore, we recommended adding Wheatley's trichrome staining to the differential diagnosis workup of cases of non-specific chest infections, especially when BPL is suspected, to avoid overdiagnosis or underdiagnosis of it.

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