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Different Staining Methods in Diagnosing Lophomonas blattarum in Bronchoalveolar Lavage Samples

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ABSTRACT

Introduction:
Lophomonas blattarum is a multi-flagellate protozoan that causes bronchopulmonary infection in humans. As the culture and molecular diagnosis of *Lophomonas blattarum* have not yet been developed, direct slide examination from nasopharyngeal secretions and bronchoalveolar lavage (BAL) is the best method for the detection of Lophomonas with morphological features. In the present study, to achieve quick and easy identification of Lophomonas, the sensitivity of different staining techniques was compared with the direct wet slide as the gold standard. Giemsa, Trichrome, and Papanicolaou stained slides have been examined in patients who had lophomoniasis.

Materials and Methods:
The BAL samples of patients suspected of lophomoniasis were collected. After confirmation of *Lophomonas blattarum* by observation in the direct test, the slides were prepared using Giemsa, Trichrome, and Papanicolaou staining for each patient.

Results:
Among the 158 BAL specimens sent to the laboratory of Imam Reza Hospital, Mashhad University of Medical Sciences, Mashhad, Iran, 50 samples were positive by direct microscopic examination that were stained by Giemsa, Trichrome, and Papanicolaou techniques. The highest sensitivity was seen for Papanicolaou staining with 16%, followed by Giemsa and Trichrome staining with 12% and 8%, respectively.

Conclusion:
The findings of the present study indicated that the Papanicolaou staining technique had the best sensitivity, compared to Giemsa and Trichrome stained slides for differential diagnosis of this protozoan from epithelial cells.

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Introduction

Lophomonas blattarum is a multi-flagellated protozoan that leads to bronchopulmonary lophomoniasis (BPL) (1). The hindguts of termites and cockroaches are considered as the main inhabitation of this parasite. The trophozoites can change to cystic form in feces in external environmental conditions. Human bronchopulmonary infection occurs by inhalation of dust containing *L. blattarum* (2,3).More than 100 cases of BPL have been reported in the published papers that have presented non-specific clinical signs, including cough, fever, and breathlessness. It has been revealed that 35% of patients with lophomoniasis demonstrated eosinophilia in laboratory tests (4).

The molecular diagnosis of *L. blattarum* has not been developed and the culture is difficult rather than other parasites found in the cockroach gut (5).

However, several techniques are implemented to identify this parasite, among which, direct examination under light microscopy is a common diagnostic method. Since morphological features of ciliated bronchial epithelial cells are most similar to *L. blattarum,* they have to be considered in the differential diagnosis (6).

Creola bodies, ciliated bronchial cells in small groups, and ciliocytophthoria, degenerative ciliated cells with fading of the stria and cytoplasmic debris are misdiagnosed as multi-flagellates in the stained smears.

The frequent samples sent to the lab for the diagnosis of *L. blattarum* include bronchoalveolar lavage (BAL), sputum, tracheal aspirates, and bronchial brushings. The candidate stains for BAL samples for diagnosis of parasitic infections are likely trichrome, Giemsa, Gram, and hematoxylin and eosin (6,7). Subsequently, regarding the high prevalence of this protozoan in the region, the present study compared Giemsa, trichrome**,** and Papanicolaou staining methods to improve the diagnosis of *L. blattarum* in the stained BAL specimens, differentiate this multi-flagellate protozoan from respiratory epithelial cells, and evaluate the staining methods in comparison to fresh smears.

Materials and Methods

Ethical considerations

The Ethics Committee of Mashhad University of Medical Sciences approved this research proposal with the Ethical Code of no.IR.MUMS.fm.REC.1395-599 in agreement with Helsinki Declaration guidelines. The patients who signed the consent forms participated in this study. The parents signed a guardianship agreement for children to attend this research.

Parasite material and design of the study

The BAL samples were collected from 150 patients with bronchopulmonary infection who were suspected of lophomoniasis and referred to the laboratories of Ghaem, Imam Reza, and Dr. Sheikh hospitals, Mashhad University of Medical Sciences, Mashhad, Iran, within April 2015-March 2016. Different slides were prepared for wet direct examination and staining with Giemsa **,** trichrome**,** and Papanicolaou.

Staining procedures

The different slides from each patient were stained after drying. Papanicolaou staining was performed according to Koss protocol, including fixing in 95% ethanol, rinsing in water (10 dips), placing in Gill's hematoxylin for 1 min, soaking in water until the slide is clear, soaking in 0.5% ammonia water for 1 min, washing in water and then in modified orange G for 1 min, two stages of 95% ethanol (10 dips each), Eosin Azure 50 for 1 min, two stages of 95% ethanol (10 dips each), two stages of 100% ethanol for 2 min in each step, two stages of xylene for 2 min in each step, and finally, two stages of placing the slides in xylene for 5 min in each time (8).

The Giemsa staining was carried out using absolute methanol for 30 sec to fix slides and left to dry. In the next step, the slides were immersed in 20% fresh Giemsa solution for 20 min (phosphate buffer with pH 7.2) (9).

Trichrome staining was performed according to Garcia protocol (10). After fixation in Schaudinn's for 30 min, the slides were located 5 min in 70% iodine ethanol, and subsequently, 5 min in 70% ethanol for twice, 10 min in trichrome stain, and 1-3 sec in acetic-acid-alcohol (90% ethanol); soaked in 100% ethanol; placed 6 min in 100%

ethanol during two stages; and finally, soaked 10 min in xylene during two steps (5 min each).

Evaluation of different staining techniques

Different staining mounts and wet slides of BAL samples were observed by microscope Olympus (×400 and ×1,000 magnification). Photographs were taken using a Tucsen TrueChrome Metrics Scientific Camera. The different staining methods were compared concerning the quality of stain and the visualization ability for flagella and cellular contents with direct slide as a gold standard.

Data analysis

The sensitivity, specificity, and positive and negative predictive values were interpreted and compared to fresh slides in SPSS software (version 24) using the receiver operating characteristic curve. The obtained data were statistically analyzed using Pearson's Chi-square with a 95% confidence interval.

Results

In this study, 50 out of 158 BAL samples sent to the laboratory of Imam Reza Hospital were positive by direct microscopic examination, which were stained with three staining methods (Giemsa, trichrome, and Papanicolaou). Analysis of data demonstrated sensitivities of 16%, 12%, and 8% for Papanicolaou, Giemsa, and trichrome, respectively. All results are demonstrated in figures 1, 2, 3, and 4 and tables 1 and 2.

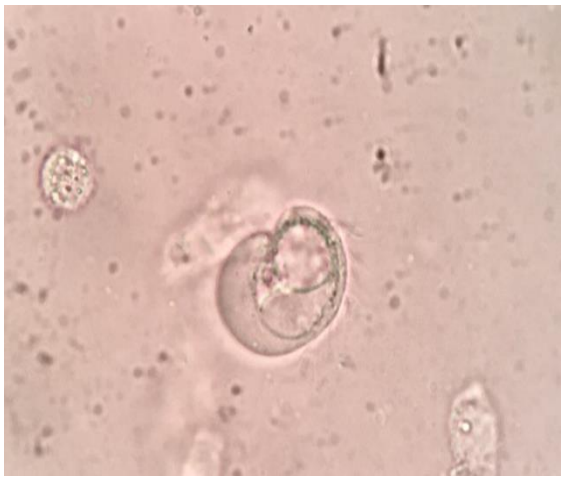

Figure 1: *Lophomonas blattarum* in wet mount microscopy of bronchoalveolar lavage fluid sample (×1,000 magnification)

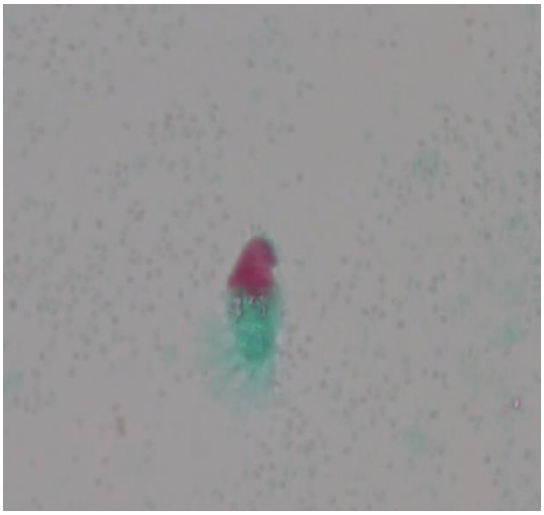

Figure 2: *Lophomonas blattarum* in Trichrome staining of BAL fluid sample (×1,000 magnification)

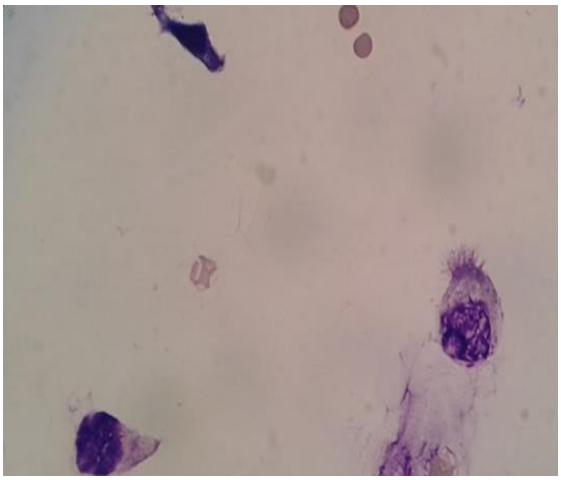

Figure 3: *Lophomonas blattarum* in Giemsa staining of bronchoalveolar lavage fluid sample (×1,000 magnification)

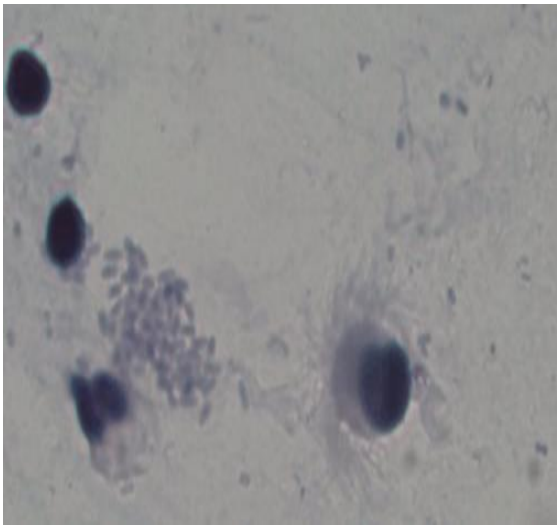

Figure 4: *Lophomonas blattarum* in Papanicolaou staining of bronchoalveolar lavage fluid sample (×1,000 magnification)

Table 1. Results of Giemsa, tri-chrome, and Papaniculau staining methods and direct microscopic examination for the diagnosis of *Lophomonas blattarum* in bronchoalveolar lavage samples

Staining of bronchoalveolar lavage samples		Results of different methods	Sensitivity
		No. (percentage)	
Direct microscopic	Pos. Neg.	50 (100%) 0	Gold standard
Giemsa	Pos. Neg.	6(12%) 44(88%)	12%
Trichrome	Pos. Neg.	4(8%) 46(92%)	8%
Papanicolaou	Pos. Neg.	8(16%) 42(84%)	16%

Table 2. Comparison of different staining methods for diagnosis of *Lophomonas blattarum*

Staining method	**Color of *Lophomonas blattarum***	**Staining quality**	**Comment**
Wet mount	No color	Best quality	Wavy movement and undulating flagella
Giemsa	Purple	Good quality	Good stained but hazy
Trichrome	Green	Good quality	Vesicular nucleus good stained
Papanicolaou	Purple	Very good quality	Vesicular nucleus very good stained

Discussion

Clinical symptoms of BPL are not completely known. Researchers believe that BPL should be considered among patients with eosinophilia, immunosuppression, and failure antimicrobial therapy (11). A few studies have investigated the differential diagnosis of *L. blattarum* from respiratory epithelial cells with closely mimicking morphological features.

Although respiratory epithelial cells are different in content and form, misdiagnosis of them frequently occurs with *L. blattarum*. In the present study, different staining techniques were investigated to achieve the most efficient method for the diagnosis of *L. blattarum* (6).

In this study, 50 out of 158 BAL samples sent to the laboratory of Imam Reza Hospital were positive by direct microscopic examination, which were stained with three staining methods.

A negative result for direct slides cannot exclude lophomoniasis since no other applicable method has been established with acceptable sensitivity and specificity. The sensitivity of direct microscopic examination is completely dependent on the technical expertise of the observer. Furthermore, in low numbers, *Lophomonas* can be easily missed especially when they lose their motility for cooling at room temperature and other flagellate parasites. Therefore, the patients' samples should be examined as fast as possible (7).

Analysis of data demonstrated sensitivities of 16%, 12%, and 8% for Papanicolaou, Giemsa, and trichrome, respectively. This result showed that the sensitivity of staining methods was not enough to diagnose the parasite, in comparison to direct microscopic examination. Contrary to this finding, Alam-Eldin et al. investigated various methods of staining in order to a better and more accurate recognition of *L. blattarum* and concluded that trichrome staining was better than others in quality and demonstration of parasite contents (6). It could be dependent on the quality of stains and the staining protocols implemented to diagnose.

Lophomonas is morphologically differentiated from epithelial cells by a light microscope. This parasite is observed spherical-elliptically in shape with granular cytoplasm, large vacuoles, and irregular flagella on the anterior side in the direct method. This is consistent with the results of a study carried out by Martinez et al. showing that *Lophomonas* might be identified by samples derived from sputum, bronchus, and BAL, in the direct and staining methods (4). In another study, the Papanicolaou staining technique was used to diagnose *Lophomonas*. The findings of the mentioned research indicated that morphologic characteristics, such as round shape, lack of load terminal, and polar flagella, could be observed well in this method (11).

It is easy to identify the terminal bar and basal nucleus of the epithelial cells in stained samples that makes it less probable to be mistaken with *Lophomonas*. On the other hand, regarding the color deposition in the field of smears, the plasticity of this protozoan, different life stages of forms, inability to determine motility, and distortion of cell size/shape lead to more confusion in identification in the stained smears. Whereas, respiratory epithelial cells are columnar form. The previous studies also reported similar results. Alam-Eldin et al. demonstrated that the nucleus of *L. blattarum* was not usually visible (6).

Conclusion

Although the staining methods could achieve a better observation of protozoan content, direct slide examination of BAL samples with high sensitivity was the best diagnostic method for *L. blattarum*, in comparison to staining techniques. Moreover, the direct method needs less time and cost to faster and simpler diagnosis.

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