

Comparison of Papanicolaou and Acridine Orange stains in the Diagnosis of Trichomonas Vaginalis Infection in Vaginal Discharge

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ABSTRACT

Background: Trichomonas vaginalis is the most common cause of vaginitis. Diagnosis based on clinical presentation is often inaccurate. Clinical presentation with adjuvant laboratory evidence of trichomoniasis is required for the confirmation of the diagnosis and initiation of therapy. The aim of the present study was to compare the sensitivity and specificity of rapid staining Acridine Orange method for detection of trichomonas vaginalis infection with the routinely employed Papanicolaou staining.

Materials and Methods: Vaginal swab smears were prepared from 300 patients attending gynaecology outpatients' clinic of S.S.G. Hospital, Vadodara with complain of abnormal vaginal discharge. The prospective study was conducted over a period of two years (from January' 98 to December' 99). The Papanicolaou and Acridine Orange stained smears were screened for presence of Trichomonas vaginalis infection.

Results: In the present study, the incidence of Trichomonas vaginalis infection was 12.3%. Considering Papanicolaou stain as standard, the sensitivity and specificity of Acridine Orange (AO) stain for detection of T. vaginalis was 92.8% and 93.01% respectively. The positive and negative predictive values were 74.2% and 99.2%, while false positive and false negative values are 3% and 0.66% respectively.

Conclusion: Trichomonas vaginalis infection is a sexually transmitted disease of the reproductive age group, which needs to be timely diagnosed to prevent its adverse effects. The rapidity, ease and reliability of Acridine Orange stain justify its use in routine laboratory diagnosis of Trichomonas vaginalis infection.

Keywords: Trichomonas Vaginalis, Papanicolaou Stain, Acridine Orange Stain, Fluorescence

Introduction

Trichomonas vaginalis is the most common sexually transmitted disease (STD) worldwide and affects an estimated 170-180 or more million people annually.^[1,2] The WHO estimates an incidence of 276 million new cases each year.^[3] Trichomoniasis is almost entirely a disease of the childbearing era. There is no doubt that this infection is sexually transmitted. Nevertheless, the male carrier is generally symptom free, because a certain amount of oestrogenic activity is required for the trichomonads to reach full maturity. In some instances, the infection can be acquired by inadequate hygiene or the use of infected towels, toilet seats, douche nozzles, clothes, bed linen and contaminated swimming pool water.^[1] Iatrogenic spread occurs through use of contaminated gloves and improperly sterilized surgical instruments especially vaginal speculum. Reinfection can occur from untreated sexual partner, extrvaginal site lodgment of parasite, which is inaccessible to local therapy and, urinary tract.

Incubation period of Trichomonas vaginalis (T. vaginalis) varies from 5 to 28 days.^[4] 75% patients complain of profuse yellowish-white, greenish-yellow to creamy-white vaginal

discharge. It is usually thin, but up to 30% cases have thick discharge. 7-10% patients complain of foul smelling discharge. Pathognomonically the discharge is foamy or frothy due to fermentation of carbohydrates, which is seen in about only 10% of patients.^[5,6] The presence of secondary infection may mask the self-diagnostic sign of frothy discharge. Burning and irritation of vulva becomes worse after periods due to increased levels of oestrogen, which favors T. vaginalis growth. Infection may be associated with genital pruritus and pain (dyspareunia, dysmenorrhea, lower abdominal pain and lower backache). It may cause urinary symptoms like dysuria, frequency and/or urgency.

Since the introduction of metronidazole for the treatment of trichomoniasis, a major problem in the control of this disease has been the accuracy of diagnosis. Also number of strains resistant to metronidazole is on an increase.^[5] And there is no alternative treatment currently.^[3] It is so widespread and causes so much ill health among young women, that it has become an important social problem. In addition to abnormal vaginal discharge, it is increasingly associated with endometritis during pregnancy, pre-term labor, premature rupture of membranes, abortion, post-

cessarean infection, and human immunodeficiency virus (HIV) infection.^[4,7] *T. vaginalis* is associated with 1.52-fold increased risk of HIV-1 acquisition.^[8] Therefore, the detection of *T. vaginalis* infection is of utmost importance. The elimination of trichomoniasis depends not only on treatment of the symptomatic cases, but also in detecting carrier patients who have mild symptoms or none, or who accept their vaginal discharge as 'normal' because every woman has a discharge anyway.

Wet smear examination though simple is an insensitive method. A minimum concentration of 10⁴ organisms per milliliter of vaginal discharge is necessary for wet mount to be positive.^[2-7] The acknowledged insensitivity is in partly due to the rapid loss of the characteristic motility of the microorganism. A large number of culture and staining techniques have been designed to increase the reliability of laboratory diagnosis of *T. vaginalis*. Diamond's medium is considered the gold standard. More recently culture method composed of liquid medium is used.^[5] Culture is a sensitive method for detecting trichomonas as compared to wet smear preparation, however it is relatively slower, requiring 2-7 days for diagnosis.^[4]

Papanicolaou stain, Giemsa stain, and other Romansowsky stains are amongst the stains for vaginal smears commonly used for the diagnosis of *T. vaginalis*.^[2] It is difficult to diagnose when trichomonads are scanty. Rounded forms of trichomonads can be confused easily with polymorphonuclear leukocytes or may look like dysplastic cells. Bare nuclei, degenerated cells and inspissated mucous plugs can be confused with trichomonads. The need for more sensitive, specific rapid and simpler method for detecting trichomonas vaginalis infection, to overcome the drawbacks of the existing methods, was recognized for a long time. More recent reports, describe the use of Acridine Orange stain using fluorescence microscope. The stain is differently adsorbed by the parasite and holds promise in improving the diagnostic ability of trichomonas vaginalis infection.

The present study is undertaken to evaluate the usefulness of Acridine Orange staining in the diagnosis of vaginal trichomoniasis and compare the efficacy with Papanicolaou staining routinely employed on vaginal smears. To define the performance characteristic of Acridine Orange stain, Papanicolaou stain is considered as standard for the present study.

Materials and Methods

The present study was carried out in the Department of Pathology, Medical College and S.S.G. Hospital, Vadodara,

Gujarat, India over a period of two years (from January '98 to December '99). 300 patients attending gynaecology clinic of S.S.G. Hospital with complain of abnormal vaginal discharge formed the 'target group' of this prospective study. A detailed clinical history was taken. The findings were noted down in the performa.

Smear Collection: *Trichomonas vaginalis* infection is essentially of the vaginal epithelium. The parasites shelter between rugae and acquire their food from glycogen rich vaginal epithelium. The specimen for the present study was thus collected from the posterior vaginal fornix pool. The vaginal swab containing the specimen was rolled onto two clean slides, one for Papanicolaou stain and other for Acridine Orange. The slides were immediately fixed by immersing in a coplin jar full of cytofix (a mixture of equal volumes of 95% ethanol and ether).

Processing of Smears: The smears were fixed in cytofix for a minimum of 30 minutes and stained by Papanicolaou and Acridine Orange. In case of any delay in staining, the slides are removed from cytofix and air-dried. For Acridine Orange stain, the staining characteristics are better with cytofixed slides than air-dried smears.

Papanicolaou (Pap) Staining Method:

90% ethyl alcohol	10 dips
70% ethyl alcohol	10 dips
50% ethyl alcohol	10 dips
Running water	1 minute
Harris's haematoxylin	2 minutes
Running water	2 minutes
Acid alcohol	1 dip
Running water	5 minutes
50% ethyl alcohol	4 dips
70% ethyl alcohol	4 dips
90% ethyl alcohol	4 dips
Orange G. 6 (O.G. 6)	5 minutes
95% ethyl alcohol	4 dips
95% ethyl alcohol	4 dips
Eosin azure 50 (EA 50)	2 minutes
95% ethyl alcohol	4 dips
100% ethyl alcohol	4 dips
100% ethyl alcohol	4 dips
Xylol	half an hour
Xylol	half an hour
Blotted and mounted with Distrene 80 Dibutylphthalate Xylene (DPX)	

Microscopic Appearance of *T. Vaginalis* Infection in Pap Smear: In Papanicolaou smears, the parasites appear as gray-green, round or elliptical structures, varying in size

from 8 to 20 μm (Fig. 1). Large size trichomonads appear when growing conditions are unfavorable while smaller sizes prevail in favorable growing conditions.

The smears from trichomoniasis are usually rich in leukocytes. There is excessive cytolysis. Debris and large plaques of necrotic cells are common. These make the background appear 'dirty'. Clustering of leukocytes and macrophages around epithelial cells, called the 'cannon ball or BB shot appearance', may be observed. There is excessive eosinophilia involving the normally basophilic cells such as the intermediate and the parabasal (Fig. 2). Leptothrix, which are long filamentous bacterial structures, commonly occur in conjunction with trichomonads. All the above features are clue to the diagnosis of trichomonas vaginalis infection, however identification of the parasite is essential before the diagnosis can be established with assurance.

It may be difficult to find *T. vaginalis* in severe inflammation (Fig. 3). Inspissated material (Fig. 4) and bare nuclei (Fig.5) may be confused with *T. vaginalis*.

Acridine Orange (AO) Staining Method:

80% ethyl alcohol	4 dips
70% ethyl alcohol	4 dips
50% ethyl alcohol	4 dips
Distilled water	4 dips
1% acetic acid	4 dips
Distilled water	1 dip
Working solution of AO (0.01%)	3 minutes
Phosphate buffer (pH 6.4)	1 minute
M/10 Calcium chloride	30 seconds
Phosphate buffer (pH 6.4)	wash
M/10 Calcium chloride	30 seconds
Phosphate buffer (pH 6.4)	dip

Smears were allowed to dry. Stained slides keep no longer than 24 hours at room temperature and 2 months at 4° C. The stock solution does not keep for more than 6 months.

Table 1: Pap and AO stained smears positive for *T. vaginalis* infection.

		Papanicolaou stain		
		Positive	Negative	
Acridine Orange	Positive	a=26	b=09	35
	Negative	c=02	d=263	265
		28	272	Total=300

a = true positive, b = false positive, c = false negative, d = true negative

Microscopic Appearance of *T. Vaginalis* Infection

Using AO Fluorescence Dye: Prior to screening, the AO stained smears are mounted in buffer. The exciter filter of the fluorescence microscope was adjusted to 400 nm and barrier filter was used as a combination of filters 44 (10% transmission at 440 nm) and 53 (10% transmission at 530 nm). Acridine Orange is a nucleic acid stain, staining differently DNA and RNA, the former fluoresces yellow to green and the latter bright red under ultraviolet illumination. Acridine Orange is very sensitive for picking up organisms. *Trichomonas vaginalis* trophozoites stain a characteristic brick red with a yellowish-green banana shaped or rounder nucleus (Fig. 6). The cytoplasm of leukocytes does not stain but the nuclei fluoresce bright green. Yeasts stain red. Bacteria including those within phagocytic cells stain bright red.^[2] The likelihood that the additional positives detected by Acridine Orange stain would not be so, is slight, since nothing else in smears significantly resembles trichomonads to cause difficulty.

Results

In the present study, comparing results by both methods in each case, 26 cases were positive for *T. vaginalis* infection by both methods. There was disparity in 11 cases, in which 2 cases were positive by Papanicolaou stain and not by Acridine Orange, while 9 cases were positive by Acridine Orange but negative by Papanicolaou stain. Combining both, there were 37 positive cases in all as shown in table 1. Incidence of *Trichomonas vaginalis* infection was 12.3%.

Considering Papanicolaou stain as the standard for present study, the sensitivity and specificity of Acridine Orange (AO) stain for detection of *T. vaginalis* is calculated as 92.8% and 93.01% respectively. Positive and negative predictive values are 74.2% and 99.2% respectively, while false positive and false negative values are 3% and 0.66% respectively.

Table 2: Incidence of *Trichomonas vaginalis* in female vaginal discharge as observed in different studies.

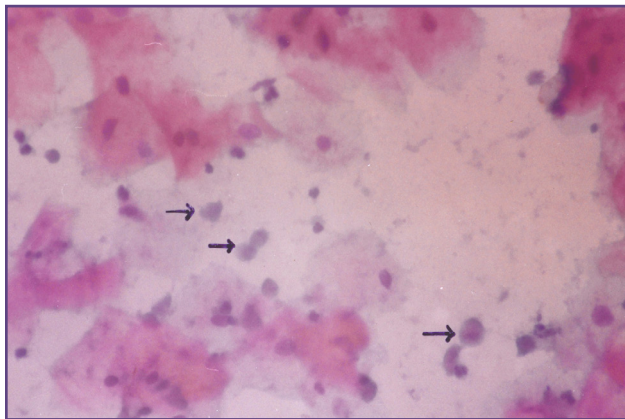
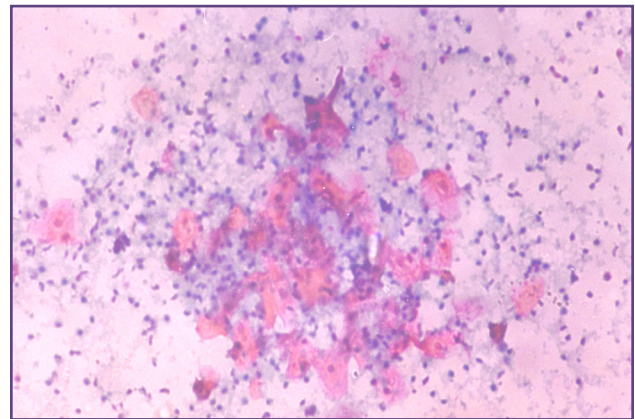
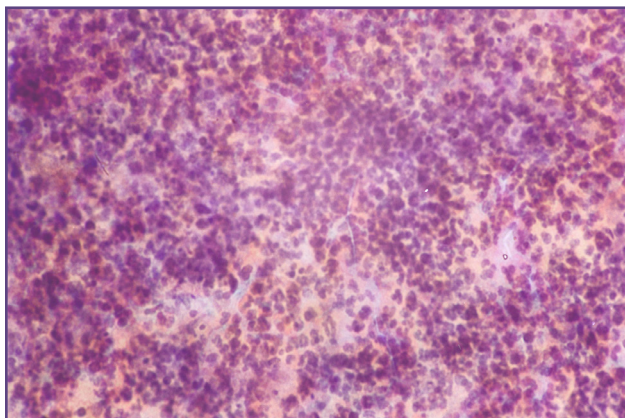
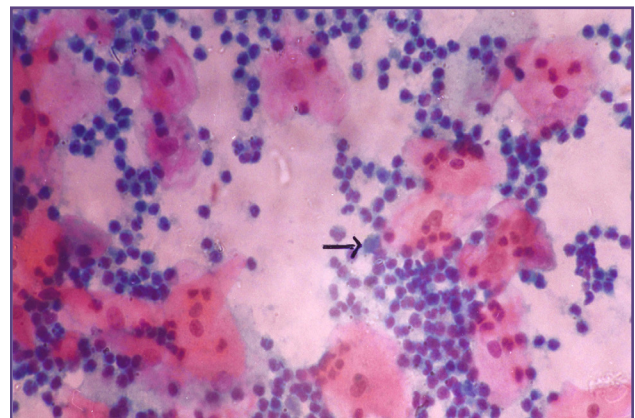
Sr. No.	Work done by	Incidence (%)
1.	Karaman et al ^[9]	8.1
2.	Klinger EV et al ^[10]	10.7
3.	Rassjo EB et al ^[11]	8.0
4.	Menezes CB et al ^[12]	15
5.	Khamees SS ^[13]	26.8
6.	Present study	12.3

Table 3: Sensitivity of Acridine Orange stain for detecting *T. vaginalis* infection.

Sr. No.	Work done by	Sensitivity (%)
1.	Khatoon R et al ^[2]	100
2.	Radonjic IV et al ^[14]	71.43
3.	Hussein AH et al ^[15]	81.8
4.	Present study	92.8

Table 4: Specificity of Acridine Orange stain for detecting *T. vaginalis* infection.

Sr. No.	Work done by	Specificity (%)
1.	Khatoon R et al ^[2]	98
2.	Radonjic IV et al ^[14]	99.44
3.	Hussein AH et al ^[15]	100
4.	Present study	93.01

**Fig. 1: (Pap stained vaginal smear, 40x view): *Trichomonas vaginalis* in Papanicolaou stained vaginal smears.****Fig. 2: (Pap stained vaginal smear, 10x view): Cannon ball appearance, dirty background and excess eosinophilia.****Fig. 3: (Pap stained vaginal smear, 40x view): Severe inflammation making it difficult to find the trichomonads.****Fig. 4: (Pap stained vaginal smear, 40x view): Inspissated material can be confused with *T. vaginalis*.**

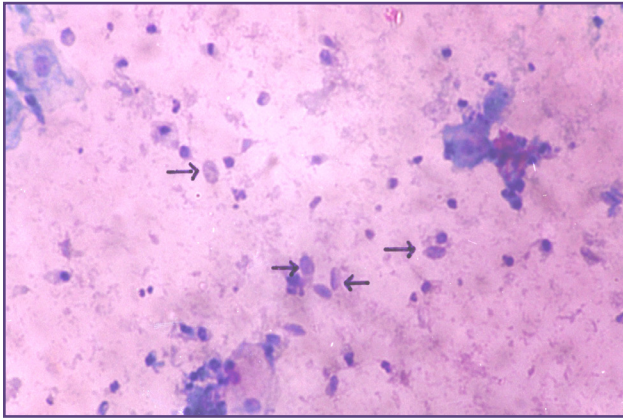


Fig. 5: (Pap stained vaginal smear, 40x view): Bare nuclei can be confused with *T. vaginalis*.

Discussion

Long considered a minor sexually transmitted disease (STD) with few associated complications, *T. vaginalis* is now known to be associated with pregnancy related complications and morbidity in non-pregnant women. Besides, trichomoniasis can be asymptomatic in nearly 50% of the infected women or they may present with wide variety of symptoms. Once established, the infection may persist for long periods in women.^[6]

As per the published literature, the prevalence of *T. vaginalis* ranges from 0.4-27.4% in women.^[4] In India, trichomoniasis accounts for 2-7% of all sexually transmitted infections.^[6] In the present study, Acridine Orange stained smears picked up 11.6% positive cases while Papanicolaou stain picked up 9.3% positive cases. Combining both results there were 37 positive cases in all, thus an incidence of 12.3%. Table 2 shows the incidence of *Trichomonas vaginalis* infection as observed by different workers.

The sensitivity of Acridine Orange stain in detecting *Trichomonas vaginalis* infection as observed by different workers is put in table 3. The sensitivity of Acridine Orange stain as observed by different workers varied between 33-93.8%. The wide range of sensitivity could be attributed to sampling error, faulty staining technique and mild infection. Nassef NE et al showed 86.7% sensitivity by AO stain as compared to culture.^[7] The specificity of Acridine Orange stain in detection of *Trichomonas vaginalis* as observed by workers is put in table 4.

Accurate diagnosis of *T. vaginalis*, whether symptomatic or asymptomatic, is affected by many variables like patient factors, clinician's experience, specimen sampling and processing and expertise of those who perform microscopy. Traditional wet mount smear is rapid method

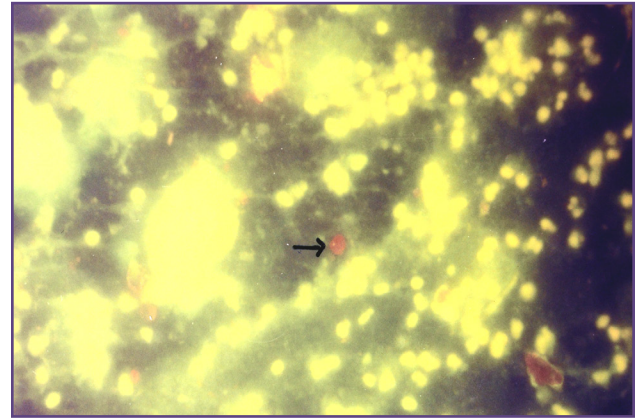


Fig. 6: (AO stained vaginal smear, 40x view): *T. vaginalis* in Acridine Orange stained smears.

but with low sensitivity and specificity. Besides it cannot be performed in clinics with heavy patient load. Cultures considered as gold standard are relatively slow. In order to identify *T. vaginalis* by culture, it is necessary to have an inoculum of 300–500 trichomonas/ml.^[14] Giemsa stain is not appropriated since it stains only the nucleus of the organism.^[12] Papanicolaou stained smear examination needs competence and expertise. False-positives as well as false negatives are common with this technique.^[3] Cellular picture of inflammatory vaginal discharge is similar particularly in trichomonas and Chlamydia. Acridine orange staining has the advantage of ease and rapidity to perform with higher sensitivity and specificity for diagnosis of *Trichomonas vaginalis* infection. The only disadvantage is the requirement of special microscope and availability of the dye. However, the cost of fluorescent microscope has decreased, so simplicity and speed of AO staining and diagnosis should encourage its' use.^[14] AO stained smears lose their fluorescence; hence it is not possible to have permanent record. Latex agglutination, enzyme-linked immunosorbent assay, immunochromatography-based tests, direct immunofluorescence assay, DNA hybridization probe test and polymerase chain reaction (PCR) are the newer tests.^[14, 7, 3]

Conclusion

High prevalence, increasing resistance to treatment and association with health complications are the burning issues related to *T. vaginalis* infection. A definitive diagnosis of trichomonas vaginalis infection cannot be made on clinical symptoms alone. Diagnosis must be laboratorial. Considering the higher sensitivity, specificity and the rapidity and ease of Acridine Orange staining, its use in routine laboratory diagnosis of trichomoniasis is justifiable. This will provide early diagnosis and prompt treatment to patients suffering from the infection. It will

help in reducing disease associated morbidity and adverse health outcomes. Although, in gynaecologic practice the value of Papanicolaou stained smears cannot be under estimated because of its primary usefulness in screening women for cervical cancer, but Acridine Orange staining is advocated in cases suspected of trichomonas infection.

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