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Short communication

COMPARISON OF INPOUCH TF KITS AND DIAMOND'S MEDIUM FOR *TRICHOMONAS GALLINAE* IDENTIFICATION IN DOMESTIC PIGEONS

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ABSTRACT

Trichomonas gallinae parasitizes a variety of avian orders especially in domestic pigeons (Columba livia domestica, Order Columbiformes). A variety of methods have been reported for the identification of *T. gallinae* in pigeons. Diagnosis of *T. gallinae* was accomplished by microscopic examination of oropharyngeal swabs of pigeons (n=120, males=78, females=42) using microscopic examination of InPouch TF culture kits and cultures taken form modified Diamond's medium during various incubation time periods after post inoculation (PI). Total 68% pigeons were found positive for *T. gallinae* by InPouch TF kit and 55% were with modified Diamond's medium. Significant differences were found between incubation time of for InPouch TF kit (CI-99%, p=.001) and for modified Diamond's medium (CI-99%, p=.000<α). No significant sex-related difference in diagnosis of *T. gallinae* was found for InPouch TF kit (p=.839) and for modified Diamond's medium (p=.765). InPouch TF as more sensitive method for the diagnosis of *T. gallinae* infections was also found to be practically efficient than Diamond's medium in recovering the parasite from specimens and may provide a readily available, low-cost substitute for the Diamond's medium in Pakistan.

Key words: InPouch TF kits, Diamond's medium, Diagnosis.

INTRODUCTION

Trichomonas gallinae (Sarcomastigophora) is a zooflagellate which parasitizes a variety of avian orders and causes avian trichomoniasis. There are various morphologic forms such as pyriform (Tasca and De Carli, 2003) round, stalked amoeboid and bell-shaped amoeboid. The bell-shaped amoeboid form has been shown to cause cell damage to avian palatal-esophageal epithelium during disease onset and progression (Kietzmann, 1993). This protozoan is pear-shaped and measures about 7–11 µm (Mehlhorn *et al.,* 2009). It also has four anterior flagella and an axostyle. *T. gallinae* is not found in the cyst form (Levine, 1961). *Trichomonas gallinae* is considered one of most serious pathogen in pigeons all over the world causing high mortalities and massive losses in bird fauna, especially in columbiformes (Work and Hale, 1996; Villanua *et al.,* 2006). Domestic pigeon, as the main host of *T. gallinae* plays an important role in the spread of this parasite among other members of the order and causes morbidity and mortality.

Despite of its significance to health of columbiformes, limited information of the prevalence of *T. gallinae* infecting domestic pigeons is available all over the world including Pakistan, because there are only limited investigation of this pathogen affecting domestic pigeons reported (McKeon *et al.,* 1997; Dovc *et al.,* 2004; Padilla *et al.,* 2004; Luo *et al.,* 2006; Sansano-Maestre *et al.,* 2009; Zhang, 2009). Different methods have been reported for the identification of *T. gallinae*. Diagnosis of infection can be accomplished by direct smear method (wet mount), Wright-Giemsa staining technique and microscopic examination of material scraped from the oral cavity of infected birds (Stabler, 1951; Coles, 1980; Anderson *et al.,* 2009; Qiu *et al.,* 2012) or by the inoculation of such material culture on Diamond's medium (Kocan and Knisley, 1970; Honigberg, 1978; McKeon *et al.,* 1997). InPouch TF kits (BioMed Diagnostics, California; Bunbury *et al.,* 2005; Bunbury, 2011) have also been used for identification of *T. gallinae*. Hofle *et al.,* (2004) also identified flagellated protozoa as *T. gallinae* by polymerase chain reaction (PCR) in woodpigeons (*Columba palumbus*) in southwestern Spain. However, very few studies have been done for the comparison of sensitivity of all these methods. The aim of current research was to compare the sensitivity of the two mentioned methods as well as finding most appropriate technique identifying *T. gallinae* (Rivolta, 1878) in domestic pigeons, in Lahore, Pakistan.

MATERIALS AND METHODS

Rearing Pigeons: Total 120 apparently health pigeons were purchases from local bird fanciers. All pigeons were tagged with different colored rings, cage-reared in the animal house of Department of Zoology, Govt. College University Lahore, Pakistan and supplied with commercial complete feed and corn (Qiu *et al.,* 2012).

Grouping: All 120 pigeons were categorized according to their gender i.e. 78 males and 42 females (Tables 1). After measuring weight in grams using manual weight

balance, pigeons were placed in four different categories of weight. The lowest range of weight was 181 grams and highest was 380 grams. Among those 14 pigeons were laying between 181-230 grams, 40 belonged to 231-280, 38 belonged to 41-55 weight group, while 28 were in category of 331-380. (Table 2)

Diagnostic Technique

InPouch TF Kit: All subjects were oropharyngeal swabbed for presence of trichomonad parasites using imported InPouch TF kits (BioMed Diagnostics, San Jose, USA). The InPouch culture kit consists of a double chambered plastic pouch separated by a narrow channel. It acts as a cell culture for parasites as well as a transparent chamber for immediate observing of sample which is equivalent to the wet mount method. The lower chamber is filled with 4 ml of a medium containing antibiotics and a fungal growth inhibitor (Kimsey, 1986). The culture medium contains reagents like trypticase, peptone, yeast extract, maltose along with other sugars, amino acids, inorganic salts, antimicrobial and antifungal drugs dissolved in normal saline phosphate buffer. Inoculated InPouch TF kits were incubated at 37^0C for 72hr and were examined under microscope (250 x magnification) for the presence of *T. gallinae* every after 24hr (Parker *et al.,* 2003; Bunbury *et al.,* 2005, 2011). An inoculum of 1 to 10 organisms is enough to diagnose a positive test if the specimen is inoculated immediately after collection. InPouch kits which were remained free of trichomonad activity after 3 days of incubation were recorded as negative.

Modified Diamond's medium: Swabs taken from the oropharyngeal region were inoculated in modified Diamond's (TYM) medium. Bacto Agar (0.5 g) can also be added to original recipe of the medium which improved the isolation and growth but was not strictly necessary so we did not include it. Following ingredients were then dissolved in 600 ml of the distilled water. 20.0 g of trypton, 10.0 g of yeast extract, 5.0 g of maltose, 1.0 g of L-cysteine hydrochloride, 0.2 g of ascorbic acid and adjusted pH to 6.0 with 1N HCl. Solution was brought to 1000 ml with distilled water and autoclaved for 15 min at 121 °C under 15 lb. /in^2 pressure to be sterilized. Then solution was dispensed in 10 ml quantities in 12 ml screw cap tubes. Each tube was supplemented with 0.5 ml antibiotic mixture Sodium penicillin G, Streptomycin sulfate, Grisiofulvin and 1 ml inactivated fetal calf serum. Sterile cotton swabs containing sample were inoculated in tubes containing modified Diamond's medium (Diamond, 1957; Kocan and Knisley, 1970; Honigberg, 1978; McKeon *et al.,* 1997; Tasca *et al.,* 1999; Parker *et al.,* 2003). Cultures were observed up to 72 hrs (three consecutive days) to check the growth of live motile parasites (Sansano-maestre, 2009).

Incubation time: After the inoculation of swabs in InPouch TF kits and modified Diamond's medium modified, kits and culture vials were placed in the incubator 37^0C (± 0.5). Cultures were observed to check the growth of live parasites (Sansano-maestre, 2009) for the time intervals of 24 hours of post inoculation (PI) i.e. after 24 hours, 48 hours and 72 hours. InPouch kits and cultures which were diagnosed positive for *T. gallinae* were isolated from which were remained negative until 72 hours. After the 72 hours, those kits and cultures were recorded as negative which showed no trichomonad activity.

Statistical tools: The efficiency of different techniques were compared by using Chi square in SPSS for Windows (Release 16.0 standard version, SPSS Inc., Chicago, USA) (Villanua *et al.,* 2006).

RESULTS AND DISCUSSION

Incubation Time: We have found significant differences (*CI-99, p=.014*) between incubation time of for InPouch TF kit (*CI- 99, p=.001*) and for modified Diamond's medium (*CI- 99%, p=.000<α*). *Figure 1* is given to show different incubation times and the number of positive samples by InPouch TF kit and modified Diamond's medium.

InPouch TF kit: We have found 82 (68.3%) of the samples of swabs taken, positive for *Trichomonas gallinae* the number of samples found to be negative is 38 (31%) (*Figure 2).* According to our results, among 42 female pigeons 28 were found to be positive and 14 negative by InPouch TF kit. On the other hand 54 males were positive and 24 were found to be negative in the diagnostic test (*Figure 3*). We found no significant difference between gender groups and InPouch TF kit as *p=.773>α*. The results showed significant difference between weight groups for number of positive samples diagnosed by the InPouch TF kits *(p=.001<α). Figure 4 i*s showing the frequency of positive/negative samples by InPouch TF kit among different weight groups.

Modified Diamond's Medium: We have found 66 (55%) of the samples of swabs taken, positive for *T. gallinae* the number of samples found to be negative is 54 (45%) (*Figure 2).* According to our results, among 42 female pigeons 20 were found to be negative and 22 positive by Diamond's medium. On the other hand 44 males were positive and 34 were found to be negative in the diagnostic test (*Figure 3*). We found no significant difference among gender groups for Diamond's medium as *p=.672>α.* The results showed significant difference between weight groups and number of positive samples diagnosed by the Diamond's medium (*p=.000*). *Figure 5* is showing the frequency of positive/negative samples by Diamond's medium among different weight groups.

Comparison of Diagnostic Techniques: Our results have shown that among diagnostic tests for identification of *Trichomonas gallinae*, InPouch TF kit was found to be more sensitive as compared to Diamond's medium. We have found much significant difference between the InPouch TF kit and modified Diamond's medium because $p=.006<\alpha$.

Trichomonas gallinae, since it affects avian livestock is considered as an economically major pathogen, and imposes a considerable threat to the protection of endangered species of columbiformes (Silva *et al.*, 2007). *T. gallinae* is a ubiquitous pathogen of columbiformes; infection is mostly asymptomatic but occasionally virulent strains of *T. gallinae* cause high mortality among affected domestic pigeons. Of the 120 birds examined in our study, we found 82 (68.3%) of the samples of swabs taken, positive for *T. gallinae* with InPouch TF kits (Cover *et al.*, 1994; Bunbury *et al.*, 2005). On the other hand 66 (55%) of the samples of swabs taken found to be positive for *T. gallinae* modified Diamond's medium (De Carli *et al.*, 1979). In contrast to Cover *et al.*, (1994), very significant difference between the InPouch TF kit and modified Diamond's medium was found.

The time was taken to diagnose *T. gallinae* by different tests after the post incubation of samples for 24hrs, 48hrs and 72hrs respectively. We have found significant differences between incubation time for InPouch TF kit and for modified Diamond's medium. Regarding the efficiency of the diagnostic tests, incubation time showed significant association between these techniques at $p<0.05$ (Cover *et al.*, 1994).

Of 78 pigeons in males, 54 males were positive with InPouch TF kit. On the other hand 44 males were positive by Diamond's medium. Regarding 42 females, 28 were found to be positive InPouch TF kit and 22 positive by Diamond's medium. Although we have found more males positives as compare to females but found no significant sex-related difference in diagnosis of *T. gallinae* (Gulegen *et al.*, 2005; Villanua *et al.*, 2006; Begum *et al.*, 2008) between InPouch TF kit, and modified Diamond's medium at $p>\alpha$. These show discrepancy with Al-Sadi and Hamodi (2011) who proposed males as more susceptible for *T. gallinae.*

Regarding the weight of animals, we found 11%, 33%, 31% and 23% of pigeons in 181-230, 231-280, 41-55 and 331-380 in the respective categories of weight in grams. We have found statistically significant differences between weight (grams) of pigeons and InPouch TF kits and Diamond's medium $p>\alpha$.

Table 1 showing the frequency of pigeons among two different genders.

Gender	*F*	Percent
Females	42	35.0
Males	78	65.0
Total	120	100.0

Table 2 showing the frequency of pigeons among different weight groups measured in grams.

Weight in Grams	*F*	Percent
181-230	14	11.7
231-280	40	33.3
281-330	38	31.7
331-380	28	23.3
Total	120	100.0

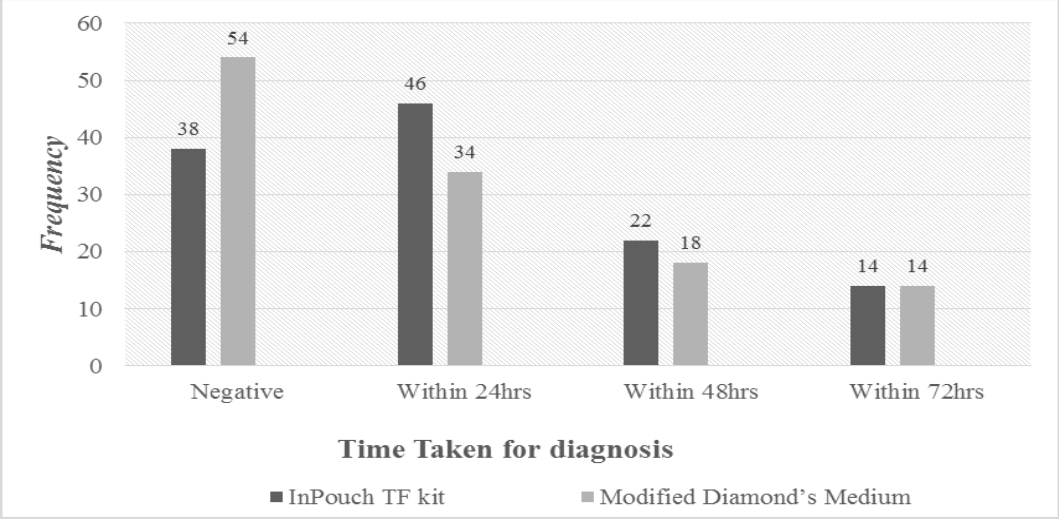


Figure 1 showing the incubation time taken by different diagnostic methods for detection of T. gallinae

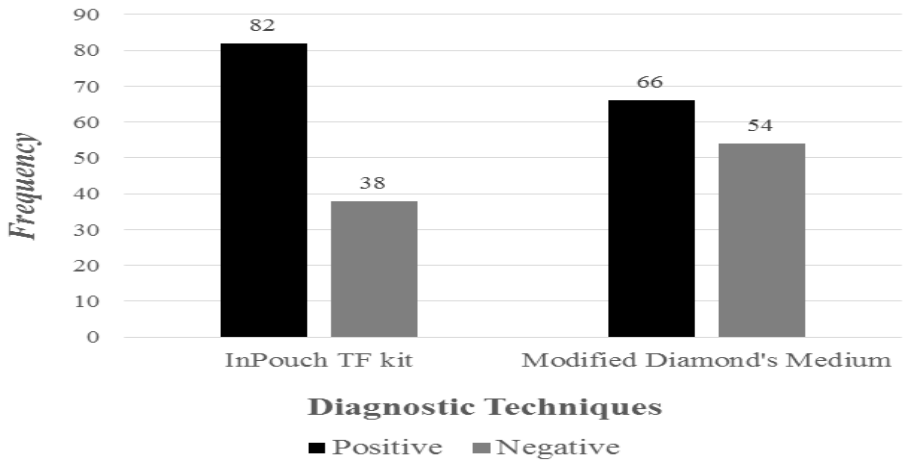


Figure 2. Showing the number of positive samples diagnosed by InPouch TF kits and Diamond's medium.

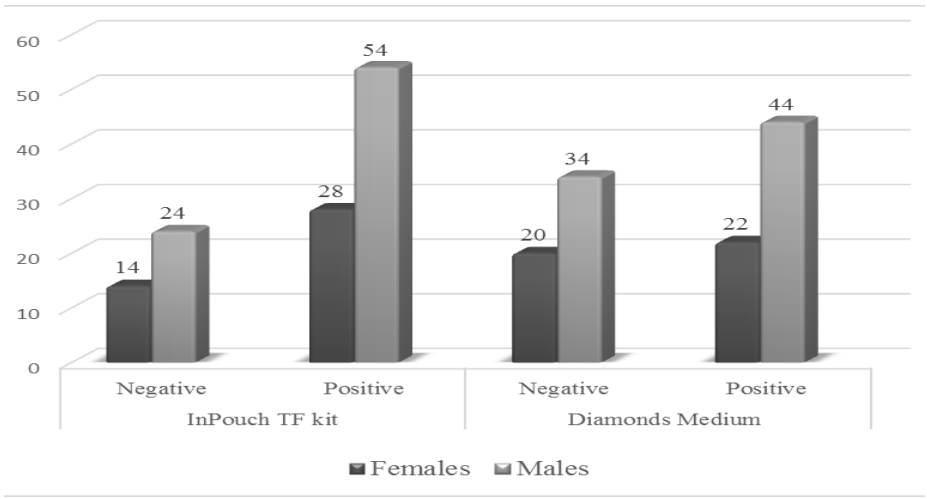


Figure 3 showing the frequency of positive/negative samples by In Poch TF kit and Diamond's medium among two gender groups

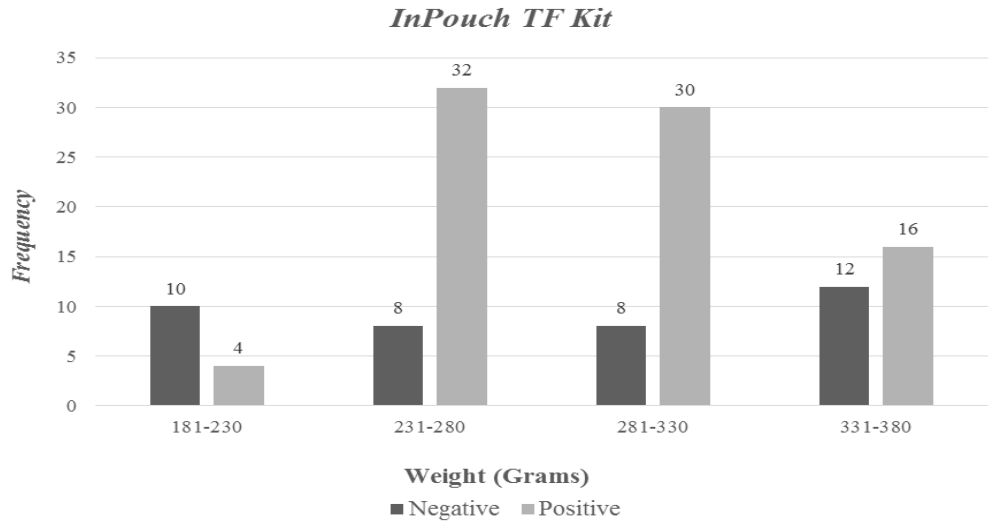


Figure 4 showing the frequency of positive/negative samples by InPouch TF kits among different weight categories

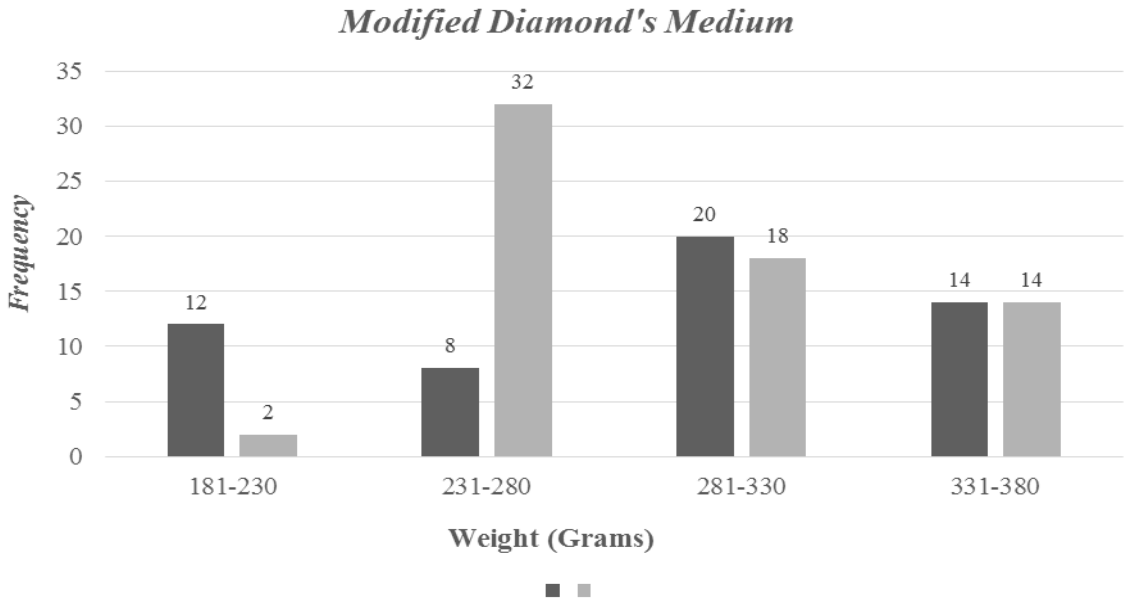
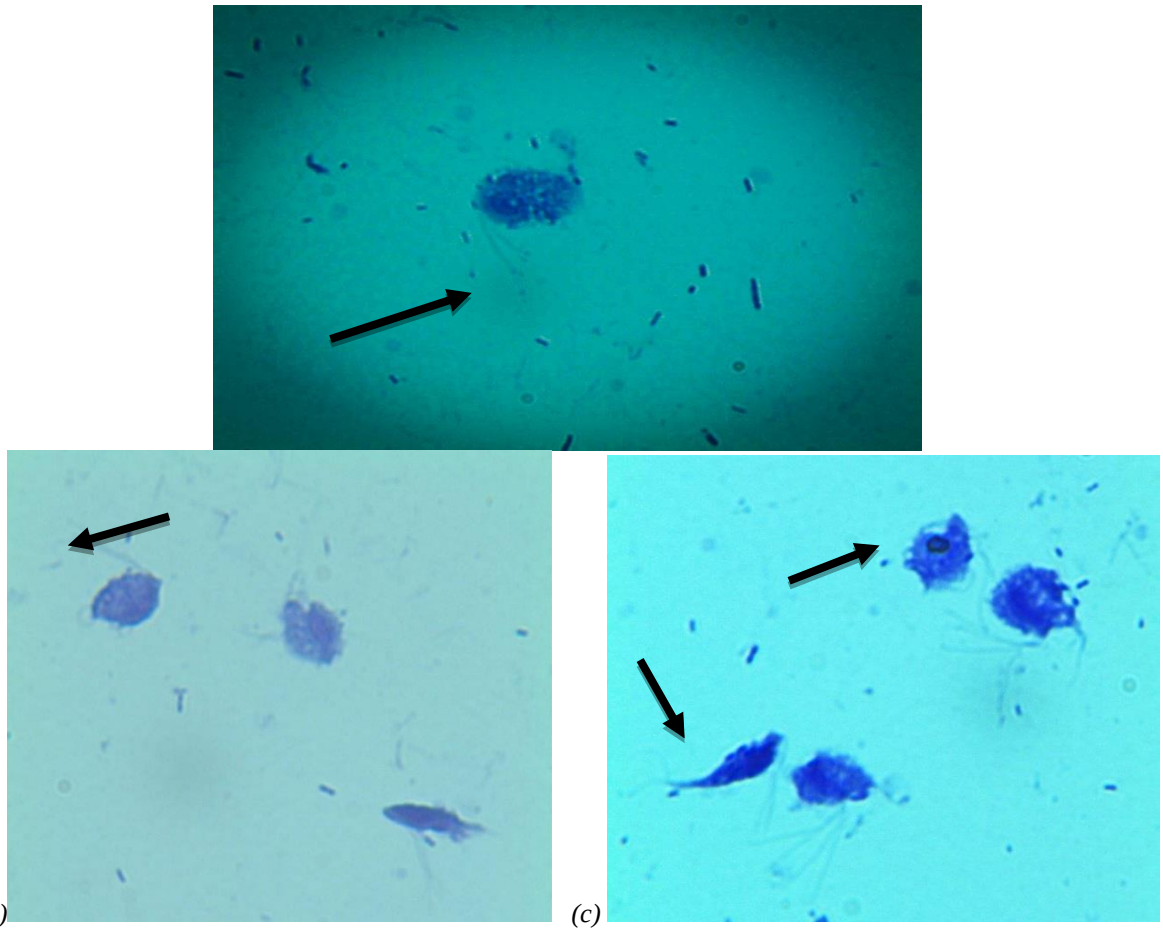


Figure 5 showing the frequency of positive/negative samples by Modified Diamond's Medium among different weight categories



(b) *(c)*

Micrographs showing Trichomonas gallinae after staining with Geimsa 1000X. (a) Arrow showing nucleus (b) Arrow indicating flagella and (c) Upper arrow indicating nucleus and lower downward indicating Trichomonas is moving position.

Conclusions: Diamond's medium is considered as "gold standard" for identification *T. gallinae* in animals. On the other hand, InPouch TF kit has plenty of benefits over Diamond's medium used in glass vials. Pouches neither break nor easily spill out. The pouch is quite lighter and easier to handle in the field than a usual glass culture tube in the laboratory. Another advantage is that, if pouches are kept vertically after inoculation, protozoans are concentrated by gravity at the bottom of the pouch which enhances the identification of trichomonads. Unlike culture tubes of Diamond's medium, once inoculated, an InPouch is not needed not be reopened for repeated examinations. Also, aseptic technique in examining established cultures is not required. The one year long shelf life at room temperature of InPouch allows the convenient keeping of a supply of pouches. So, it's more expenditure than the mount microscopy could be acceptable for these advantages along with its high sensitivity and potentially lower subjectivity in diagnosing infectious organisms.

Ultimately, we propose InPouch TF as more sensitive method as compared to Diamond's medium which makes it as perfect technique for the diagnosis of *Trichomonas gallinae* in avian fauna especially the fancy birds in field conditions as well as in the laboratory.

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