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Original Article

Chlamydia and Vaginitis in Sexually Active Females: Classical Identification Methods for Effective Control

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ABSTRACT
Laboratory diagnosis of *Chlamydia* and vaginitis in sexually active females has been limited by unavailability of a sequential method/rapid technique for simple diagnosis. Six hundred (600) adult females from hotel/brothel, Sexually Transmitted Infections (STIs) Clinic, Obstetrics/Gynaecology Clinic, Family Planning Clinic and Healthy controls were investigated for *Chlamydia*, *Candida*, trichomoniasis and bacterial vaginosis (BV). This was done using microscopy: wet mount, stained vaginal secretion and stained smear after culture. Results showed that there were 72% infections in the female groups. The brothel and STI group had infection in the range (70-86%). Chlamydial infection was highest in the STI group while *Candida* infection was highest in the healthy (control) females. Bacterial vaginosis was distributed in all groups. As *p*-value increased, *f*-value increased indicating constant co-infection of *Candida* and BV in Chlamydia positive females. Microscopy by direct detection from sample and stained smear after culture were in the range: 56-86%. Direct microscopy for BV was 78.5% and stained smear after culture, 57.1%. Sensitivity and specificity of the techniques showed that detection of *Chlamydia* was less sensitive by direct microscopy of sample but sensitivity and specificity of stained smear after culture were high. Immunoassay (32.2%) was also less sensitive. Sensitivity and specificity of wet mount microscopy for *Candida*, Trichomoniasis and BV were in the range 62.5 – 80% and 62.5-97.8% respectively. Wet mount has high sensitivity and specificity for detecting agents of vaginitis and may be useful for routine use and for diagnosis where disease is absent, thus, making identification more cost effective.

Keywords: *Chlamydia*, Culture, Microscopy, Staining techniques, Vaginitis

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INTRODUCTION

Chlamydial infection and vaginitis constitute problems in the female genital tract (Cates *et al.*, 1990) and symptoms of acute vulvovaginitis remain one of the most common reasons for women's healthcare visits. *Chlamydia trachomatis* is the most common cause of sexually transmitted infection in the world (CDC, 1993). Other pathogens that make up vaginitis are *Candida albicans* (Candidiasis), *Trichomonas vaginalis* (Trichonomiasis) and Bacterial vaginosis. Bacterial vaginosis is the single entity caused by many organisms including *Gardnerella vaginalis*, *Mobilincus* spp, *Mycoplasma hominis*, *Bacteroides* spp, Gram positive anaerobic cocci (*Peptococcus* and *Peptostreptococcus*) species (Willet and Centor, 2005; Lowe *et al.*, 2009). These organisms and other sexually transmitted pathogens are associated with high risk for HIV infection (Hilber *et al.*, 2010) by causing genital lesions which facilitate viral entry or by increasing the number of target cells for HIV (activated monocytes) (Greenblatt *et al.*, 2005).
Complications which include cervicitis, urethritis,

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endomethritis and pelvic inflammatory disease (PID), increase incidence of ectopic pregnancy and infertility are common (Friedek *et al.,* 2004). Although *Chlamydia* and vaginitis are known to cause serious problems in female sexual and reproductive health (Wilson *et al*., 2005), yet diagnostic methods for *Chlamydia* are non-definitive. The use of test kits is uncertain, the tissue culture methods using McCoy cell lines are expensive and the use of embryonated hen's eggs is laborious (Stamm *et al*., 1983; Jespersen *et al.,* 2005). The non – culture method using Chlamydiazyme and Microtrak direct fluorescent antibody, Sylva Micro-trak enzyme immunoassay (EIA) using DFA, PCR and DNA probe analysis with varying sensitivity and specificity are expensive and beyond the reach of most laboratories (Black, 1997; New Hall *et al*., 1999; Lowe *et al*., 2009). These issues constitute problems in the diagnosis of *Chlamydia* and the aspect of identifying *Candida albicans*, *Trichomonas vaginalis* and Bacterial vaginosis (BV) has not been properly resolved.

Cultivation of micro-organisms has been shown to be gold standard for phenotypic identification of organisms (Jespersen *et al*., 2005). Microscopy and staining techniques have also been shown to reveal micro-organisms causing genital infections. Even deep seated infections with unknown route or origin have been deduced using the microscope. Stained smears using gram-stain and other standard stains have been used to detect and reveal intracellullar existence of *Chlamydia* (Schacter, 1990). Additionally, the sensitivity and specificity of these methods have been highlighted by research scientists (Hadgu and Stemberg, 2009), and values obtained were used to grade usefulness in diagnosis/detection of diseases.

In Nigeria, as in other African countries, the paucity of data concerning *Chlamydia* and vaginitis has created vacuum in diagnosis of these infections. Besides no such diagnostic methods are in place. Hence, it is important to have a baseline data upon which further investigations could be made and also institute straight forward combined methods for prompt diagnosis. This study therefore evaluates *Chlamydia* and vaginitis in sexually active females from various socioeconomic backgrounds. Moreover, it is aimed at providing a harnessed investigation system in the laboratory for identifying *Chlamydia* and vaginitis which invariably will be merged into a single linear procedure for proper and prompt diagnosis in our setting.

SUBJECTS, MATERIALS AND METHODS

Subjects

A total of six hundred (600) participants were randomly selected from various groups constituting females from Hotels / Brothels (250), STI clinic (100), Obstetrics /Gynaecology centre (100), family planning clinics (50) and apparently healthy undergraduates (100). The participants were from both private and government medical centres in Lagos metropolis. The laboratory investigations were carried out at the Molecular Biology Division of the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos and Infirmary Laboratory, Lagos.

Ethical Consideration

Proposal was approved by Institutional Review board (IRB) of NIMR, Yaba. Informed consent was obtained from the participants.

Sample Collection

From each participant, four swabs were collected: two endocervical swabs (ECS) and two High Vaginal Swabs (HVS). One HVS was used for microscopy and the other for culture on artificial media. One ECS for immunoassay and the other for culture in Embryonated Hen's Eggs (EHE). Samples were transported to the laboratories using Hanks transport medium (Black, 1997).

Laboratory Investigation of Samples

Wet Mount

Microscopy was carried out on vaginal extracted secretions by squeezing swab of sample in 0.5ml of normal saline for yeast cells and other organisms. For detection of Candida and *Trichomonas vaginalis*, one drop each of the extracted vaginal sample was placed on opposite sides of a clean slide. A drop of 10% potassium hydroxide was added to one of the fluid and sniffed for fishy odour (whiff test); positive reaction indicates bacterial vaginosis. Other Amsel criteria were applied to detect BV. They include detection of clue cells, pH > 4.5 (pH strip of range 4.0-7.0) of the vaginal secretion and homogenous discharge (Amsel *et al*., 1983). The second preparation was covered with a cover slip and observed for oval cells / hyphae to detect *Candida*. Jerky movement was used to detect *Trichomonas* species (Romanik and Martinosian, 2004).

Dry Mount

This screening test was done to detect pathogens directly from freshly collected samples. A drop of vaginal sample which was extracted in 0.5ml of 0.2 normal saline placed on slide and allowed to air dry. Thereafter, they were Gram stained (Nugent *et al.*, 1991). Smears were also made directly from endocervical samples before culture and stained with Giemsa for *Chlamydia* while Gram staining was applied to detect other pathogens. This was done to screen for pathogens directly from freshly collected samples.

Culture in Synthetic Media

High Vaginal Swab was cultured on various media. For *Candida*, sample was cultured on Sabouraud Dextrose Agar (SDA). For *Trichomonas vaginalis* culture was done on Cysteine Peptone Liver Maltose Agar (CPLM OXOID, Basingstoke). Culture was incubated aerobically for 24hrs at 37°C. Facultative culture of samples from female vagina was done for BV in primary isolation medium using Columbia Agar with colistin and nalidixic acid (CAN) (OXOID, Basingstoke) supplemented with 1% proteose-peptone and 5% whole human blood. This was done to identify *Gardnerella vaginalis* and other likely pathogens. Vaginal samples were swabbed and plated on CAN and incubated in carbon dioxide candle extinction jar at 37°C for (24- 48) hrs. Thereafter, growth was analysed for likely pathogens (Cowan and Steel, 1993; Romanik *et al.*, 2005). Anaerobic cultures were not done because of unavailability of some necessary reagents.

Culture in Embryonated Hen's Eggs (EHE)

Embryonated Hen's eggs (EHE) were drilled using the dental equipment (Reco-Dental, Wicsbaden, West Germany mot: 117821). After cleaning each egg with 70% ethanol, the round blade provided was used to make a cut at the apex of the egg such that the upper part of the egg formed a cap. The extracted endocervical sample in phosphate buffer saline was inoculated into the eggs by using syringes to penetrate the chorio-allantoic membrane. Egg was inoculated and sealed with a label to avoid exposure to air contaminants. Incubation was done for two weeks at 37°C. Every other day, eggs were candled and impression smears were made and stained with Giemsa. Thereafter, they were viewed for inclusion bodies. After screening eggs for dwarfening of embryo, those that looked hazy were selected and passage into another set of EHE (Schacter, 1967)

Microscopy and Analysis after Culture in various Media

After culture on various media, Embryonated Hen's Egg (EHE), Sabouraud Dexrose Agar (SDA), Cysteine Peptone Liver Maltose Agar (CPLMA) and Columbia Agar with colistin and nalidixic acid, smears of growth/colonies were made and stained with iodine (20%). Confirmatory staining was done with Giemsa for *Chlamydia* and Gram stain for other pathogens. Thereafter they were examined for *Chlamydia*, *Candida, Trichomonas* and agents of Bacterial vaginosis (Moncada *et al.,* 1990)

Immunoassay

Following manufacturer's instructions, samples were assayed using qualitative test kits, Quick-Vue (Quidel, USA) and Diaspot, (America) whereby the principles are based on Antigen-Antibody reaction. Both positive and negative test control were included in each pack.

Storage of Organisms

Strains of *C. trachomatis* were harvested into 2SP (0.2M sucrose in 0.02M phosphate buffer, pH 7.0) in 1ml amount with antibiotics (Schacter, 1990) i.e. 0.2% Streptomycin or 0.5% Erythromycin (to wade off bacterial contaminants) and frozen at -70°C in aliquots. Individual aliquots when needed were thawed immediately prior to use and cultured in 2-5P to give (10-20) inclusions per x400 field for 100μg inoculum. This will be used for typing with monoclonal antibodies in subsequent studies.

Data Analysis

This was done based on bimodal distribution of observed values. *P*-values for statistical tests of significance was by the level of alpha = 0.05 which requires a *P*-value to be less than 0.005 before considering difference to be statistically significant. Sensitivity and specificity estimates were obtained by considering Embryonated Hen's egg culture as "gold standard".

RESULTS

Microscopy of Stained smears from Vaginal Secretion and Smear of Growth after Culture

Positive *Chlamydia* stained with Giemsa was shown by characteristic brownish-purple cytoplasmic inclusion (Figure 1).

Candida appeared as small (2 to 4 μm), oval or budding yeast like cells and pseudomycelial element which were strongly gram positive. *Trichomonas vaginalis* appeared as oval shaped with pointed apex. Gram stain of vaginal secretions from women without bacterial vaginosis showed a predominance of gram positive bacilli (Lactobacilli) and a few other bacteria. Those from women with BV showed large numbers of small, gram-negative coccobacilli (*G. vaginalis*). About half of the cases had gram-negative to gram variable, thin curved rods consistent with *Mobilincus species.*

Table 1: Infection Rate among Category of Women Enrolled in the Study

Location	Number tested	Number with infections (%)	*Chlamydia* (%)	*Candida* (%)	*Trichomonas* (%)	Bacterial Vaginosis (%)
Brothel/ Hotel	250	212(85)	30 (14)	70 (33)	12 (6)	100 (47)
STI Clinic	100	70 (70)	15 (21)	20(29)	5 (7)	30 (43)
Obstetric and Gynecology	100	61 (61)	10 (16)	30 (30)	1 (2)	20(33)
Family Planning Clinic	50	43 (86)	5 (12)	15 (35)	3 (7)	20 (47)
Healthy Group/ Undergraduates	100	47 (47)	2(4)	25 (43)	0 (0)	20 (43)
Total	600	433 (72)	62 (14)	160 (37)	21 (5)	190 (44)

$F = 6.82$; $P < 0.05$`, $X^2=35.03$

Table 2: Detection Rate of *Chlamydia* and Agents of Vaginitis by various Methods

Microbial Pathogens	Direct Microscopy of stained Samples (%)	Immuno-assay (%)	Wet Mount (%)	Culture (%)	Microscopy of stained smear after culture (%)
***Chlamydia trachomatis* (62)**	35 (56.4)	20 (32)	NA	50 (80.6)	28 (56)
***Candida albicans* (160)**	100 (62.5)	NA	120(75)	105 (65.6)	60 (57)
***Trichomonas vaginalis* (21)**	18 (85.7)	NA	18(85.7)	15 (71)	86.6%
Bacterial vaginosis (190)	110(57.9)	NA	130(68.4)	140 (73.68)	80 (42.1)

NA= not applicable

Relative Distribution of *Chlamydia* and Other Infections

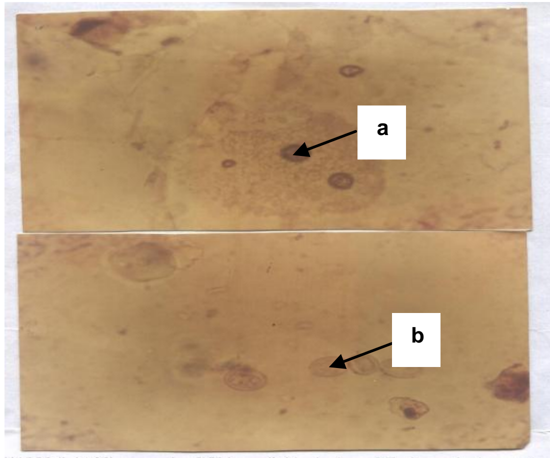
There was a net distribution of 72% of infection amongst the different groups. The brothel/hotel based, STI clinic and the family planning groups had 70-86% infections. Occurrence of *Chlamydia* was highest amongst those attending STI (21%) clinic, followed by brothel/hotel (14%) based females. The least for *Chlamydia* were the apparently healthy individuals.

Candidiasis was highest amongst the apparently healthy undergraduates (43%) while the Obstetrics/Gynaecology, hotel and Family Planning (FP) were in the range (30-35%). *Trichomonas vaginalis* was 7% for STI and FP females and bacterial vaginosis infections were in the range 33-47% for all female groups with the most occurring in the STI clinic and brothel groups (Table 1).

Association of *Chlamydia* with other Pathogens in the various Female Groups

The STI females who had the highest distribution of *Chlamydia* (21%) were followed by hotel/brothel based group (14%). The association of *Chlamydia* with *Candida* and BV gave the value $p < 0.05$ which was significant in the brothel/hotel and STI Clinic groups while the value for *Chlamydia* was insignificant in the control group (undergraduates). An association of $x^2 = 35.03$ for the STI and O/G groups showed that those within these categories are likely to encounter *Chlamydia and Candida*. Co-infection of *Chlamydia* with other pathogens were shown

by the significant values of $p< 0.05$ and $f = 6.82$. As *p*-value increased, *f*-value also increased showing that *Chlamydia* and other pathogens co-infect in the various symptomatic and asymptomatic individuals (Table 1). The pie chart illustration (Figure 2) shows *Chlamydia* at 50^0 (14.31%) with adjacent BV and Candida at 158^0 (43.87%) and 133^0 (36.31%) respectively. *Trichomonas* completed the circle with 18^0 (4.84%). This chart indicates interlocking relationship of the pathogens.



X100 Magnification Light Micrograph

a: shows inclusions containing elementary bodies or infectious particle of *Chlamydia*, with a resistant rigid envelop and metabolically inactive. b: shows inclusions containing reticulate or initial bodies of *Chlamydia*. It is metabolically active and has a fragile envelope

Figure 1: *Chlamydia* inclusions of Endocerical Sample Grown in Embryonated Hen's Egg

Comparative Detection of *Chlamydia* and Agents of Vaginitis by Various Methods

Direct detection of *Chlamydia* and agents of vaginitis showed Giemsa staining of endocervical sample, 35 (56.4%) for Chlamydia while wet mount for *Candida*, *Trichomonas* and BV revealed pathogens in the range (68.4-85.76%). Immunoassay was 32% for *Chlamydia*. Culture revealed growth of pathogens in the range (65.6-80.6%). Microscopy after staining detected 57.9% for BV and 62.5% for Candida respectively while 42.1% for BV showed incomplete culture since anaerobes were not cultivated (Table 2). Growth of *Chlamydia* in EHE yielded 80.6%, an indication that the embryonated hen's egg support intracellular existence of the pathogens (Figure 1 and 2).

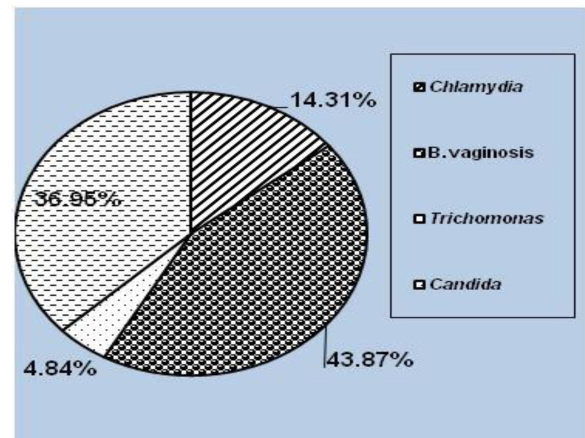


Figure 2: Distribution of *Chlamydia* and other Pathogens among Groups of Sexually Active Women

Table 3: Comparison of Identification of Chlamydia and Agents of Vaginitis Direct from Stained Samples

Direct Microscopy of Infectious	Culture		Total No	Sensitivity (%)	Specificity (%)
	Positive	Negative			
Chlamydia					
+	35	5	40	56.4%	-
-	27	233	260	-	97.8
Candida					
+	100	20	120	62.5	-
-	60	120	180	-	85.7
Trichomonas					
+	18	18	36	85.7	-
-	3	11	114	-	86
Bacterial vaginosis					
+	110	60	170	78.5	-
-	30	100	130	-	62.5

Table 4: **Comparison of Stained Smears of *Chlamydia* and Agents of Vaginitis after Culture**

Culture	Culture		Total No	Sensitivity	Specificity
	Positive	Negative			
Chlamydia					
+	28	34	62	80	-
-	7	231	238	-	87.1
Candida					
+	60	100	160	75	-
-	20	120	140	-	54.5
Trichomonas					
+	13	8	21	72	-
_	5	124	129	-	94
Bacterial vaginosis					
+	80	60	140	57.1	-
-	60	100	160	-	62.5
Immunoassay Kit: *Chlamydia*					
+	20	0	20	32.2	-
-	38	242	280	-	100

Comparison of Sensitivity and Specificity of the Various Methods and Techniques against Culture

Direct microscopy for *Chlamydia* was sensitive by 56.4% and specific by 97.8%. C*andida* was sensitive by 62.5% and specific by 85.7%. *Trichmononas* had sensitivity of 85.7% and specificity of 86% while BV was detected with a sensitivity of 78.5% and specificity of 62.5% (Table 3). Stained smears after culture for *Chlamydia* had 80% sensitivity and 87.1% specificity. *Candida* had 75% sensitivity and 54.5% specificity. *Trichomonas* had 72% sensitivity and 94% specificity. Stained smear detection for BV was 57.1% sensitive and 62.5% specific. Immunoassay detected *Chlamydia* by 32.2% sensitivity and 100% specificity (Table 3 and 4).

DISCUSSION

Although it is known that *Chlamydia* and vaginitis constitute problems of the female genital tract, the specificity of identification of causal agents often poses problems to the microbiology laboratory (Prey 1999; Franklin and Monif, 2000). Identification of *Chlamydia* was done in the early 1960's. In this earlier study, Embryonated Hen's Eggs were used and animals such as baboons (Grayston *et al.*, 1960; Schacter *et al.*, 1967) gave insights to the proper identification of *Chlamydia*. Control of bacterial sexually transmitted diseases was espoused by Schmid *et al.* (2005), who analysed the various component of vaginitis. Although the analyses were done in details to to effect prompt diagnosis. Similar findings were show the existence of such related problems in the female genital tract, but did not solve the problem of diagnosis. This present study showed a relationship between microscopy and culture and formed a strategy which combined their various identification approaches in a linear method for prompt diagnosis.

Unlike earlier studies which were based on various methods (Nugent *et al.*, 1991), the high rate recorded by direct staining of samples and wet mount microscopy in our study revealed that organisms colonising the genital areas may also become pathogenic. Agents of vaginitis colonised healthy individuals Gaur *et al.* (2010), making bacterial vaginosis a subject of controversy as to when it should be regarded as true infection. To further elucidate the problem associated with diagnosis, it was shown that the detection of *Chlamydia* in groups of women from O/G, FP, STI and healthy controls showed a gradient in rates of *Chlamydia* detected by kit (32%), microscopy (56.4%) and culture (80.6%). These findings agree with previous suggestions that laboratory results should be matched with clinical symptoms (Bradshaw *et al.*, 2005; Oakeshott *et al.*, 2006; Lowe *et al.*, 2009).

An association of *Chlamydia* with other pathogens was shown by a *p*-value which increased as *f*-value increased indicating co-infection. The significance of x^2-value is that *Candida* and BV will likely occur in *Chlamydia* positive females confirming a channel for combined tests observed for *Candida, Trichomonas* and BV as

regards rates of detection. Although, syndromic treatment of non-gonococcal urethritis, mucopurulent cervicitis and Pelvic Inflammatory Disease (PID) has been shown to be effective for managing those caused by *Chlamydia trachomatis* as well as agents of vaginitis, this approach is insensitive since a large number of asymptomatic individuals would not be treated (Schacter, 1990; Schacter, 1994). As shown in this study, candidiasis was highest in asymptomatic females; the healthy (control) group. Thus supporting the findings of Loeffelholz and colleagues (1992), which recommended laboratory based screening of asymptomatic females.

The recorded high percentage of infection in the brothel/hotel and STI groups were related to the increased rates of detection by microscopy. The observed rates for *Candida albicans* and bacterial vaginosis as shown by microscopy of wet mount, revealed the shapes of the pathogens which are indices for detection. Microscopy of stained endocervical secretion for *Chlamydia* showed inclusions (56.4%) while culture (80.65%) was most revealing. Although growth /colonies were recorded in cultivation media but they were non-specific as microscopy after staining revealed the actual organism. Culture requires further biochemical tests for confirmation of organism, this may not be necessary for routine diagnosis except for research purpose. Our findings however showed that there were no significant difference between direct microscopy of fresh samples and results of stained smear after culture except for bacterial vaginosis which had 57.9% direct microscopy and 42.1% stained smear after culture. This was probably due to lack of anaerobic culture. Therefore, direct microscopy of sample may be substituted for culture in routine laboratory analysis (Willet *et al*., 2005).

The sensitivity of culture for *Chlamydia* has been estimated to range from 50 to 80% in laboratories with advanced experimental procedures (Schacter *et al*., 1994). Same applies to culture of agents of vaginitis (Hiller, 1993). In this present study, direct microscopy of *Chlamydia* gave a resolved sensitivity of 56.4% and specificity of 97.8%. It is intermediate in screening high risk group but it is specific and useful for diagnosis of negative cases; those that do not have the disease. Stained smear after culture was both sensitive (80%) and specific and may therefore be useful for routine screening and detection of *Candida*. Wet mount microscopy of vaginal secretion showed sensitivity (62.5%) and specificity (85.7%) and may not be recommended for routine use but could useful for diagnosis in asymptomatic cases due to its high specificity. For *Trichomonas vaginalis*, wet mount is both sensitive and specific for detecting the pathogen and microscopy of stained colonies is also useful. As previously proposed by Romanik *et al*. (2005), the sensitivity (78.5%) for BV by wet mount depends on identification of clue cells. It is sensitive and useful for high risk group such as brothel/hotel and the STI clinic group.

Alternative tests for *Chlamydia* detection have included enzyme immunoassays, DFA and rapid antigen tests in addition to molecular assays (Johnson and Horner, 2008). The results of this study also show that communities which do not have access to sophisticated equipment and reagents could carry out simple and cost effective microscopic technique to identify *Chlamydia* and vaginitis in an ‘’All-in-one procedure”. Our findings showed immunoassay for *Chlamydia* as 32.2% sensitivity, therefore not useful for routine use. It had a specificity of 100% and therefore could be useful for diagnosis in those that do not have chlamydial infection. The wet mount is useful for *Candida, Trichomonas* and BV while stained endocervical secretions in addition to immunoassay will be useful in detecting *C. Trachomatis* in high risk group and highly specific in diagnosis in population where the disease is absent.

CONCLUSION

Microscopy of stained sample is useful for detecting *Chlamydia* for routine diagnosis and specific where disease is absent, but immunoassay is not sensitive for routine use. Wet mount has high sensitivity and specificity for detecting agents of vaginitis for routine use and diagnosis where disease is absent and therefore more cost effective for identification. Therefore for effective laboratory diagnosis of adult females who present with genital problems, this “All-in-One Combined-Procedure” is recommended as follows: for chlamydiasis and vaginitis, two forms of microscopy are required: (a) dry mount and (b) wet mount. The dry mount will require staining of endocervical sample with Giemsa stain for diagnosis of chlamydial infections; 1-4 inclusion bodies depicts mild infection; 5-10 inclusions or more depicts active infections. Observation of Gram-negative coco-bacilli, that is, *Gardnerella vaginalis* in Gram stained HVS for diagnosis is an indication for BV in clue cell positive samples.

Depending on concentration of *Candida* and trichomonads in discharge, Gram positive yeast cell and pear shaped organisms indicate *Candida* and trichomonads respectively. Wet mount is strictly for vaginitis and presence of clue cells indicates BV; yeast cells and hyphae for *Candida albicans* and jerky movement of pear shaped organisims for trichomonads.

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