Diagnosis of vaginitis in married women by microbiological and molecular methods in Tikrit City.

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Abstract

The main aim of this study was identification of most common microorganisms associated with vaginitis.

This study was performed between Feb. 2013 and Juan. 2014, involving 215 married women at 15-49 years who attending TGH and private clinic in Tikrit city; they were examined and taking high vaginal swab. For diagnosis, used Amsel's criteria, Gram staining of vaginal discharge, culture and polymerase chain reaction (PCR).

Vulvovaginal candidiasis is most common 70(32.6%) woman infected with C. albicans than BV associated G. vaginalis 18(8.4%), in our distinct. Recognize Candida spp. are not always done by culture, with presence of E. coli and S. aureus were 25(11.6%) and 16(7.4%) respectively. The most affected age was 25-29 year (19.5%). The history of abortion and PTL in those patients was at 1st and 3rd trimester of pregnancy. In this study molecular method (PCR) was used to diagnose BV associated G. vaginalis and at the sametime to diagnose Candida spp. PCR is a novel tool with excellent sensitivity and specificity especialy in fastidious M.O as G. vaginalis. By dise diffusion method showed resistance 100% to metronidazole.

We conclude that Candida vaginitis is most common in our distinct. The clue cells and measurement vaginal pH are the most suitable tests of Amsel's criteria for prediction of BV associated G.vaginalis. In addition, the use of metronidazole is not always benefit so we need further study of screening and use of newer local antibiotics and disinfectants.

Key words: vaginitis, BV, G. vaginalis, C.albicans, PCR.

Introductions

Vaginitis is an inflammatory of the vaginal mucosa or infection of the vagina, is the most common gynecological condition encountered by physicians in the office, whose incidence appears to be increasing, as estimated that 75% of women will experience at least one episode of vaginitis (1). The most common causes of vaginitis are vaginal candidiasis, Bacterial vaginosis (BV), aerobic vaginitis (AV), and Trichomoniasis (2). Although vaginitis is not serious condition in strictly medical terms, it may have discomfort and frequent visits most of women have at least one episode of it's during childbearing years (3). It is an enormous health problem in both developed and developing countries. BV has not been considered a pathogenic condition in the past, but recently it has been found to be significantly associated with obstetric infections and possess a potential threat to the fetus and newborn (4, 5). BV is characterized by overgrowth of facultative anaerobic bacteria (Gardnerella vaginalis), also anaerobic Gram negative rods (2). Amonig the other causes of vaginitis, Streptococcal B-hemolytic, E.coli derived from normal intestinal microflora and Staphylococcus aureus is named AV (5, 6). In Caucasian women the prevalence of vaginitis is 5-15%, in African &

American blacks 45-55%. In Asian around 20-30%. More than 70-75% of women are exposed to vaginal candidiasis (7). It is caused by Candida spp. causes itching, edema, erythema of the vulva, vaginal discharge of white clots & pain (8). C. albicans is responsible for 70-90% of all vaginal candidiasis while Candida glabrata is responsible for 14%. Also Saccharomyces cerevisiae is reported as a cause of vaginitis. The pathogenesis of preterm birth and other adverse pregnancy outcomes linked with reproductive tract infection remains poorly understood. Mucolytic enzymes, including mucinases and sialidases (neuraminidase), are recognized virulence factors in bacteria that cause BV, such enzyme-producing microorganisms may increase the risk of subclinical intrauterine infection during pregnancy and thus increase risks of preterm birth (9).

Materials and Methods

Patients: The study population was composed of 215 married women, at aged between 15-49 yrs old, who attending the outpatient of gynecological and obstetric clinic in Tikrit Teaching Hospital and private clinics in Tikrit province in the period from February 2013 to January 2014. Most of the patients were suffering from vaginal discharge, itching or irritation, and sometimes bad odor, dysuria and dyspareunia. The patients with diabetes mellitus, received corticosteroids drug, malignancy, vaginal bleeding, and menstrual bleeding and received antimicrobial drugs less than one week, will be excluded from this study. Control were 65 apparently healthy (no signs and symptoms).

Sample collection and treatment: By using unlubricated sterile Cusco's speculum was inserted into vaginal women and the lateral and posterior vaginal fornix were swabbed with two sterile cotton tipped applicators. One of the swab was for put in tube with sterile Amies transport media and transported to microbiological laboratory where cultured, and other swab be was for microscopic examination (wet mount and Gram stain). A PH strip placed in contact with the secretions on the speculum after it had been withdrawn measured vaginal PH. BV and AV was clinically diagnosed when presence homogeneous vaginal discharge and PH > 4.5. Amine test was performed by adding a drop of 10% KOH to the discharge on the used speculum (whiff test), on the slide (sniff test) fishy amine odor release (10). Clue cells were diagnosed by Gram staining in which vaginal epithelial cell covered with gram variable short bacilli. The patients were considered have BV when they have at least three out of four clinical signs and symptoms (11). For culture, used selective media for G.vaginalis is Columbia blood agar base, with FD056, incubated anaerobically at 37OC/48 hour in candle jar to provide 5-10% CO2. Another swab streaked the Blood agar, Chocolate agar which were incubated in microaerophilic atmosphere while MacConkey's agar, Eosine methylene blue agar, Mannitol salt agar, Pseudomonas agar and Sabouraud's dextrose agar-Chloramphenicol incubated aerobically at 37 OC/24 h., but fungi incubation may reach 72h.

The Procedure of DNA extraction from Gardnerella vaginalis:

1. 1.5 ml of a saturated culture was harvested with centrifugation for 5 min at 14,000 rpm.

2. The cell pellet was re-suspended and lysed in 200 □l of lysis buffer (40 mM Tris-acetate pH 7.8, 1 Mm EDTA, 20 mM sodium acetate, 1% SDS) by vigorous pipetting. Then put in water bath at 65OC for 5 min.

3. To remove the most proteins and cell debris, 66 □l of 5M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 14,000 rpm for 10 min at 4OC (cold centrifuge).

4. After transferring the clear supernatant into a new Eppendrof tube, an equal volume of chloroform was added, then mix on a rotating mixer for 15 min when a milky solution was completely formed.

5. Following centrifugation at 14,000 rpm for 5 min, the supernatant is then removed to another Eppendrof tube and double volume of

100% iced Ethanol was added and added 100 □l of Ammonium acetate.

6. The tubes were inverted 5-6 times gently to precipitated DNA, then centrifuged at 10,000 rpm for 3 min.

7. The supernatant from this last step discarded and 1 ml of ethanol (70%) was added, again tubes centrifuged 10,000 rpm for 3 min.

8. Repeating Step 7, and the supernatant discarded and 50 □l of distill water was added to re-suspended (dissolved DNA).

10. The stock DNA is kept frozen at (-20OC) until use.

*PCR assay: The primer used was sialidase Sial (F): ATGGAA CGTCGTTCAACGAAG and Sial(R): GATACGCGTTTTATGTCT CTTGC. The components required for Premix PCR Reaction in both G.vaginalis & Candida including of total volume was 20 □l consist of (AccuPower® PCRPreMix 2□l, Forward primer 1□l at concentration 1.25uM, Revers primer 1□l at concentration 1.25uM, DNA Tamplate 1Ml at concentration 50 ng and Sterilized deionized distilled water 15□l).

*DNA Extraction from Candida:

For direct yeast cell amplification, a single colony approximately 1 mm in diameter was picked with a micropipette tip, suspended in 5□lof sterile D.W in a microcentirfuge tube, and vortexed; then, 0.5□l of this suspension was used in PCR. The extraction of DNA performed manually according to (12). Two primers were selected, the first was to detect Candida spp.: CANIA (GAGGGCAAGTCTGGTG) and CANIB (CTGCTTTGA ACACTCTAA), and the second was to detect C. albicans from non-albicans: CALB1 (TTTATCAACTTGTTGTCACACCAGA) and CALB2 (ATCCCGCCTTACCACTACCG) in same line with (8).

The PCR products were run on 1% agarose gel and electrophoreses were done at 100 V in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator.

Results

The present study showed vaginitis, was caused by yeast infection, more prominent 46.5% than BV associated G. vaginalis 8.3%, while AV was 39%. These results were significant, (Table 3), the most age group infected with vaginitis was between 25-29 yr. (19.5%).

The present study showed women with vaginitis and BV had previous obstetrical problems such as abortion in the first trimester were 40(18.6%) case out of 215 patients, then in third trimester including PTL and still birth about 18(8.37%), but less in second trimester including 14(6.5%) case (Table 4).

The most clinical features of BV was thin homogenous vaginal discharge (115/215), then bad offensive vaginal odor and itching were (94/215) for each of one, while dysuria was (66/215), (Figure 1).

The present study showed the most sensitive test were thin homogenous vaginal discharge and vaginal pH >4.5 had 100% for each one while specificity were 50.7% and 47.2% respectively. Most sensitive and specific test was clue cells 88.8% and 95.4% respectively, (Table 5 & 6).

PPV positive predictive value, NPV= negative predictive value Frequency of

The present study showed Candida albicans was most common microorganism 32.5% which causes Candida vaginitis, while BV associated G.vaginalis was 8.3%, as well as AV was common, caused mainly by Staphylococcus aureus, E. coli, and Staph. Coagulase negative 7.4%, 6.5% and 4.6% respectively. Then Strep. pyogenes and Klebsiella spp was 3.7% for each one (Table 7).

PCR provide to be an easy and reliable method to determine the presence of yeast, a common cause of vaginal infections. The primer pair CANIA and CANIB were used in amplification of the target region of 18S rRNA gene for Candida spp., the production about 210

bp as in (Figure 3). While specific primer for C.albicans were CALB1 and CALB2 the production of amplification size between 273-280 bp (Figure 4)which did not amplified with non-albicans candida.

Discussion

Vaginitis or vaginal infections are still a major cause of morbidity, and are not reportable diseases; therefore, accurate estimates of incidence are unavailable (6). The diagnosis methods of BV and Candida vaginitis depended on the acuity of the clinician. The lack of standardized and diagnostic tools lead to misdiagnosis and consequently incorrect treatment (13). The present study showed vaginitis, was caused by yeast infection, more prominent 46.5% than BV associated G. vaginalis 8.3%, while AV was 39%. These results were significant, (Table 3). The results agreed with (14) reported BV associated G.vaginalis incidence 5.2% in non- pregnant in Baghdad, also (15), mentioned the prevalence of BV was 7% and BV associated G.vaginalis was 7.7% in Basrah, while in AL-Diwaniya city (16), reported 28.6% women affected by BV and 93.7% of them with BV associated G.vaginalis, as well as reported 32.6% with other vaginitis like Candidiasis. In Babylon province (8) mentioned in their study out of 105 vaginal sample 97 was infected with Candida vaginitis. Also, the present study accepted with (13), which showed 10-20% of women infected with BV and approximately 75% vaginal yeast infection. This difference in incidence of BV associated G.vaginalis may be attributed to the preservative relationships in the Iraqi society, in addition to that, the fact that most women in these studies were single and possibly had multiple sexual partners (17), at the same time G. vaginalis colonizes uncircumcised men more frequently (18). The most age group infected with vaginitis was between 25-29 yr. (19.5%), agreed with (16) mentioned most affected age group between 25-34 yr. was 40.5%, also with (15), reported the mean age of women with G. vaginalis infections was 25.6 yr. compared to those with no G. vaginalis 26.9 yrs. Other study showed the age ranged between 21-25 years most infected with BV associated G. vaginalis 28.2% (14). Also accepted with (19) showed Indian women infected with BV most common 26 – 30 yr. was 23%.

The present study showed women with vaginitis and BV had previous obstetrical problems such as abortion in the first trimester were 40(18.6%) case out of 215 patients, then in third trimester including PTL and still birth about 18(8.37%), but less in second trimester including 14(6.5%) case. The result accepted with (20,21), who found 95% of premature rupture membrane and PTL has BV, and treatment of BV will reduce premature rupture membrane with PTL, (Table 4).

The most clinical features of BV was thin homogenous vaginal discharge (115/215), then bad offensive vaginal odor and itching were (94/215) for each of one, while dysuria was (66/215), agreed with most of the studies (3, 15,16,22). These may attributed to the first step in pathogenesis of BV and AV is decrease in number of normal flora (Lactobacilli spp.), which are responsible for production of lactic acid by fermentation of glycogen which presents in vaginal epithelial cells then making acidic vaginal pH, so will become alkaline which is suitable for proliferation and growth other bacteria mainly G.vaginalis and other pathogenic bacteria (Figure 1).

One of the most important test used for diagnosis of BV associated G. vaginalis is Amsel's test depending on clinical features and laboratory examination. These criteria were thin and milky homogenous vaginal discharge, vaginal pH > 4.5, positive amine test which give fishy odor, presence of clue cells (22). In the present study showed the most sensitive test were thin homogenous vaginal discharge and vaginal pH >4.5 had 100% for each one while specificity were 50.7% and 47.2% respectively. Most sensitive and specific test was clue cells 88.8% and 95.4% respectively, (Table 5 & 6).

The present study showed Candida albicans was most common microorganism 32.5% which causes Candida vaginitis, while BV associated G.vaginalis was 8.3%, as well as AV was

common, caused mainly by Staphylococcus aureus, E. coli, and Staph. Coagulase negative 7.4%, 6.5% and 4.6% respectively. Then Strep. pyogenes and Klebsiella spp was 3.7% for each one. The results accepted with (15), reported Candida vaginitis was 20.4%, and G.vaginalis was 7.7% while Staph spp., and E. coli were 9.9% and 2.4% respectively. Streptococcus spp., and Klebsiella spp. were 6.1% and 1.5% respectively. Also, in the same line with (6, 16, 23), mentioned C.albicans was 11.9%, while Staph. aureus and Beta haemolytic streptococci were 7.4% and 3.6% respectively (16).

PCR provide to be an easy and reliable method to determine the presence of yeast, a common cause of vaginal infections. The primer pair CANIA and CANIB were used in amplification of the target region of 18S rRNA gene for Candida spp., the production about 210 bp as in (Figure 3). While specific primer for C.albicans were CALB1 and CALB2 the production of amplification size between 273-280 bp (Figure 4)which did not amplified with non-albicans candida (8).

Regarding G. vaginalis produce certain virulent factors such as Sialidases, formerly known as neuraminidases, are enzymes which cleave alpha-ketosidic linkages between the glycosyl residues of glycoproteins, glycolipids. Sialidases have been implicated as virulence factors in G. vaginalis (but not in all biotype so some G.vaginalis sialidase +ve and other -ve) demonstrated that 84% of women with BV have detectable sialidases in their vaginal fluid and that the specific activity is higher in these women than in women without the syndrome (9). We used specific primer Sial (F): ATGGAACGTCGTTCAACGAAG and Sial (R): GATACGCGTTTTAT GTCTCTTGC. The expected DNA fragment of 682 bp was observed (24).

Conclusion

Many genital infections occur because of new sexual practices, involving significant microbiological aspects. When we compared between the cultural procedures with clinical tests, we found that isolation of G. vaginalis with less efficiency and less validation in diagnosis of BV. We think the presence of clue cells with vaginal pH measurement are the most suitable tests for prediction of BV. The molecular technique (PCR) is the excellent sensitive and specific tool to identify the pathogenic organisms involved in BV and yeast vaginitis, since the last one can not be accurately diagnosed by clinical symptoms and culture to identify the Candida spp. The use of metronidazole not always useful especially those with recurrent vaginitis and BV, because of exaggerated use of metronidazole in Iraq.

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Table 1: PCR conditions used in the present study in G. vaginalis.

Steps	NO. of cycle	Characters	Temp./OC	Time
Step 1	1 Cycle	Initial denaturation	94	3 min
Step 2	30 Cycle	Denaturation steps	94	30 sec
		Annealing steps	54	30 sec
		Extension step	72	1.30 min
Step 3	1 Cycle	Final Extension	72	10 min
Step 4	Hold	--------	4	-------

Table 2:PCR conditions used in the present study in Candida.

Step	Character	Temperature	Time	Cycler
1	Initial denaturation	95OC	30 sec.	1 cycle
2	Denaturation	95OC	5 min.	30cycle
	Annealing	56OC	1.5min.	
	Extension	72OC	1 min.	
3	Final extension	72OC	10 min.	1 cycle
4	-------	4OC	-------	Hold

Table 3 :Distribution of BV and vaginitis in married women.

Disease	Patient NO.	%	Control NO.	%
C. vaginitis	100	46.51	0	0
AV	84	39.06	0	0
BV	18	8.37	0	0
Other aetiology	13	6.04	0	0
Total NO.	215	100	65	100

XX X2=280, df= 4, P value < 0.05 significant

Table 4: Obstetrical problems in patients with BV & vaginitis

Obstetrical problems	Patients No.	Controls No.
Abortion 1stb trimester	40	8
Abortion 2nd trimester	14	1
Abortion 3rd trimester	18	2

Table 5: Frequency of Amsel's criteria test prediction of BV (G.vaginalis) [Amsel et al 1983, at least 3 of 4 to consider BV].

Amsel's criteria		BV +ve culture for (G.vaginalis)	BV -ve culture for (G.vaginalis)	Total
PH	>4.5	18	104	122
	<4.5	0	93	93
	Total	18	197	215
Amine test	+	12	82	94
	-	6	115	121
	Total	18	197	215
Clue cells	+	16	9	25
	-	2	188	190
	Total	18	197	215
Thin homogeneous vaginal discharge	Yes	18	97	115
	NO	0	100	100
	Total	18	197	215

Table 6: Validity of Amsel's criteria diagnosis of BV (G. vaginalis)

Amsel's criteria	Sensitivity %	Specifity %	False Positive	False negative	accuracy	PPV (precision)	NPV
Clue cells	89	95	5	11	95	64	99
Amine test	67	58	42	33	59	13	95
T.H.vaginal discharge	100	51	49	0	55	16	100
PH	100	47	53	0	52	15	100

Table 7: Microorganisms isolated from women with vaginitis &control

Microorganism	Patient = 215		Control = 65	
	NO.	%	NO.	%
Candida albicans	70	32.6	0	0
Candida glabrata	10	4.7	0	0
Sachrmycis cervisia	20	9.3	0	0
Gardnerella vaginalis	18	8.4	2	3.07
Escherichia. coli	25	11.6	1	1.53
Staphylococcus aureus	16	7.4	0	0
Staph.Coagulase negative	15	7.0	4	6.15
Streptococcus pyogenes	8	3.7	0	0
Enterococcus faecalis	6	2.8	5	7.69
Streptococcus agalactiae	6	2.8	14	21.53
Klebsiella spp.	8	3.7	0	0
Enterobacter spp.	6	2.8	0	0
Proteus spp.	3	1.4	0	0
Pseudomonas spp.	1	0.5	0	0
Enterococcus faecium	1	0.5	2	3.07
Enterococcus avium	2	0.9	0	0
Aer. viridans	2	0.9	0	0
Streptococcus salivaris	1	0.5	0	0
NO growth of M.O.	13	6.0	37	56.92
Total	231		65	100

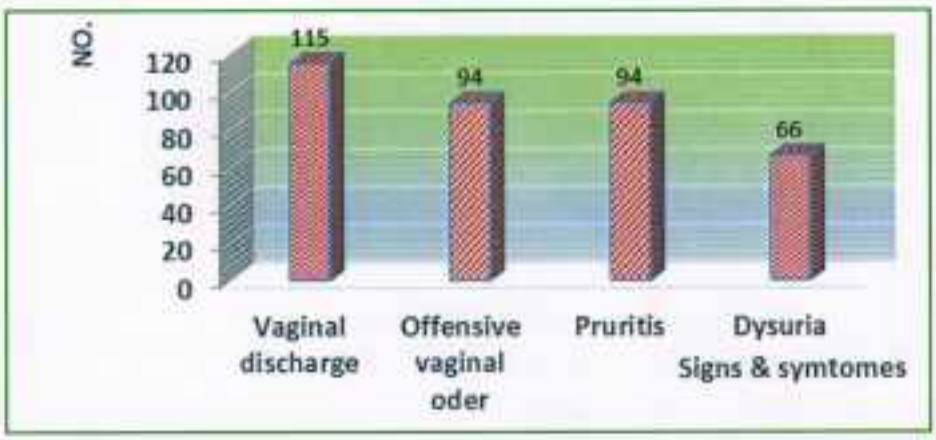

Figure 1: Genital signs & symptoms in women with vaginitis & BV.

Figure 3: Agarose gel electrophoresis of Amplified PCR products of Candida by CANIA and CANIB primer, A lane 100 bp of mlocular size.

Figure 4: Agarose gel electrophoresis of Amplified PCR products of Candida albicans by specific primers CALB1 and CALB2.