Prevalence of Extended-Spectrum Beta Lactamase producing gram negative bacteria from vaginal infection

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Abstract: ESBL producing gram-ve microbial outbreak in bacterial vaginosis is more critical especially during pregnancy. The purpose of this study is to determine the isolate and identify the ESBL antibiotic sensitivity patterns and resistance patterns of the isolates identified from vagionosis cases. A total of 200 high vaginal swabs (HVS) were obtained from pregnant (155 no) and non-pregnant (45 no) womens. The highly positive isolate was further subjected to antibiotic sensitivity tests, and minimal inhibitory concentration tests and Extended-spectrum -lactamase (ESBL) determination tests such as the combined disc method and double disc synergy test were used to investigate resistance patterns, followed by genotypic analysis to knockout resistance genes. Ambler class A beta lactamase genes such as TEM (404 bp), SHV (294 bp), and CTX-M (415 bp) were used as standards. Out of 200 HVS samples, 91 samples positive for *Escherichia coli* (41nos). Among the 45 isolates, 41 isolates were highly resistant to ESBL antibiotics screened by combined disc diffusion and E-strip test. During the Minimal Inhibitory Concentration analysis, at least 36 had MIC_{50} of 2 µg/ml, MIC_{90} of 512 µg/ml and 5 were intermediate to Cefotaxime antibiotics. 50% of isolates obtained from pregnancy samples showed ESBL production. The prevalence of drug resistance genes such as TEM, SHV, CTX-M also confirmed. The current study concluded that pregnant women between the ages of 20 - 30 had a higher rate risk for ESBL producers than non-pregnants womens.

1. Introduction

Extended-spectrum Beta Lactamase enzyme hydrolysis the beta-lactum antibiotics. Bacterial vaginosis (BV) is an inflammatory condition in sexual reproductive system of womens. It is due to dysbiosis of normal floras of vagina. The infection confirmed by irritation, itching, thin grey or whitiesh discharge with fishy smell and burning sensation in vaginal region **(Xiaodi Chen, et.,al 2021,Wondemagegn Mulu.et.,al 2015) .** When the acidic balance of vagina distrupted due to douching, pregnancy, menstrual cycle, and long-time treatment condition, entry of pathogenic bacteria, fungi, viruses or parasites may trigger the normal flora and causing elevation of vaginal pH. BV causes risk of chorioamnionitis (intra-amniotic infection), spontaneous abortion, pelvic inflammatory disease (PID) and sexual transmitted diseases (STDs) in womens.

The global economic burden to treat BV is USD$4.8 billion per year (Vodstrcil et al.,2020) . Antibiotics such as tinidazole, secnidazole, clindamycin and metronidazole are in current treat (Bradshaw and Sobel 2016).But improper or prolong usage and administration emerging antibiotics reported to develop leads to drug resistance pattern by vaginal floras which may disrupt treatment protocols (Sobel and Sobel (2021),Muzny et al.,2022.Particularly, the development of antibiotic resistance by ESBL producing bacteria results in the limitations of therapeutic options as they may exhibit co-resistance to many other classes of antibiotics (Safari et al.,2015). Since the vaginal enterobacteriaceae is a real threat and there is not much information available about the colonization of ESBL producing bacteria in pregnant and non-pregnant women, this study solely focuses on the ESBL resistance vaginal enterobacteriacea from pregnant and non-pregnant women and to evaluate the phenotypic and genotypic characterization. **(Sozan M. Sharo.et.,al 2021) .** 75% of women get vaginal infections at least once in their lifetime. In non-pregnant women who have a normal immune system, bacterial infection rarely leads to a serious complication. When antibiotic resistance pathogens travel from vagina to uterus, it leads to the development of a risk factor for utrene infection. If untreated during pregnancy, it may affect the mother and baby as premature birth, low birth weight, and also cause pelvic inflamatory disease, that can increase the risk for infertility. Hence, the current study was undertaken to evaluate the ESBL and Quinolone drug resistance.

2. Methodology

2.1 Sampling

Using sterile swabs, a total of 200 vaginal samples (pregnant-155 nos and non-pregnant-45 nos) were collected from women aged 15–50 suffering due to vaginal discharge with an odour, itching or swelling on the outside of the vagina, burning during urination, and pain during intercourse. The sampling period is 2018-2021. The obtained sample vials were labelled and delivered to the study location for further examination.

2.2 Identification of gram - negative isolates

The collected swabs were inoculated on MacConkey agar, Blood agar (Hi-Media, India), and incubated at 37ºC for 24 hours. The purified cultures were morphologically and IMViC analyzis (Indole, MR-VP, Citrate, Urease) was done.

2.3 Antibiotic sensitivity test

The isolates were subjected to the Kirby-Bauer disc diffusion method carried out to determine the antibiotic sensitivity against standard antibiotics such as imipenem (10g), cefoxitin (30µg), ceftazidime (30g), ceftazidime+clavulanic acid (30µg+10µg), Aztreonam (30µg), Piperacillin, Cefozolin (30g), Gentamicin (10.g), Nalidixic acid (30g), ciprofloxacin (5µg). The assay was performed according to CLSI guidelines

2.5 Minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) are defined as the lowest concentrations of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations as the lowest concentration of an antimicrobial that will prevent the growth of an organism after subculture on to antibiotic free media. MICs are used by diagnostic laboratories mainly to confirm resistance but most often as a research tool to determine the in-vitro activity of new antimicrobials, and data from such studies has been used to determine MIC breakpoints (CLSI guidelines 2018). During this study Cefotaxime antibiotic was used.

2.4 Phenotypic determination of ESBL producers

The phenotypic determination for ESBL producing isolates was determined by the CLSI phenotypic confirmation assay or combined disc assay and confirmed by the E-strip method. Both the tests were done according to CLSI guidelines.

2.4.1 Phenotypic confirmation or combined disc assay

The standard antibiotic disc ceftazidime (CAZ) (30g) and ceftazidime/clavulanic acid (CAC) (30/10g) were used in this study. Both the antibiotic discs were placed in MHA medium containing a lawn culture of the test organism and a 20 mm gap was maintained in between the discs. The plates were incubated at 37°C for overnight. A zone diameter of ≤27mm that increased in zone size by ≥5mm by the addition of clavulanic acid was considered positive for ESBL production. *E.coli* (ATCC 25922) was used as a control.

2.4.2 Epsilometer test or E-strip for ESBL strain determination

The porous-paper based thin strip (Himedia-Ezy MIC^{TM}) was impregnated with 2 drug molecules in a concentration gradient in the reverse direction. One end of the strip is coated with ceftazidime (0.5-32mg/L of MIC test range) and the other end is with ceftazidime (0.064-4mg/L of MIC test range) and clavulanic acid (4mg/L). According to the manufacturer's instructions, the E-strip test was done. In brief, overnight grown isolates in agar plates were suspended in a 0.85% saline solution, which is equal to the 0.5 McFarland standard. The swab culture was prepared in MHA and the E-strip was placed on it using sterile forceps. After 24 hours of incubation at 37ºC, the inhibition of the ellipse zone around the strip was analyzed. ESBL positive strains are recognized when the zone ratio for ceftazidime: the value of ceftazidime in combination with clavulanic acid is greater than or equal to 8, or when no zone is obtained for ceftazidime and clavulanic acid mixtures. (Stürenburg et al., 2004). ESBL-positive strains were linked to inhibitor-resistant TEM, CTX-M, and AmpC genes (Rawat and Nair, 2010; Leverstein et al., 2002).

2.5 Genotypic confirmation of ESBL

2.5.1 Extraction of DNA and amplification of ESBL genes (Maniatis T *et al*., 1982)

To extract the DNA, the isolates were cultivated in MHA medium upto 24 hours. When colonies reach the 0.5 McFarland turbidity value TAE buffer, the inoculum was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded, and 200µl of milliQ water was added to the pellet and vortexed, and then it was kept in a dry bath at 100˚C for 10 minutes. After that, it was immediately stored at -20˚C for overnight. The suspension was spun at 10,000 rpm for 10 minutes to elute out the DNA from the supernatant.

With the help of forward primer and reverse primers of TEM, SHV, CTX-M (beta lactamases genes) (Table 1), PCR amplification process carried out in a PCR machine. The PCR conditions followed as described in Table 2. The amplified DNA material was determined in Agrose gel electorphoresis under UV illumination.

Primers	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
TEM	AACATGGGTATCAGGGAGATG	CAAAAGCGCGTAACCGGATTGG	404
SHV	CGCCTGTGTGTATTATCTCCCT	CGAGTAGTCCACCAGGATCCT	294
CTX-M	AAA AAT CAC TGC GCC AGT TC	AGC TTA TTC ATC GCC ACG TT	415

Table 1: Primers and expected size of amplicons

STEPS	TEM	SHV	CTXM
Initial denaturation	94˚C for 5min	94˚C for 5min	94˚C for 5min
Denaturation	94˚C for 30sec	94˚C for 30sec	94˚C for 30sec
Annealing	65.5˚C for 30sec	60˚C for 30sec	60˚C for 30sec
Extension	72˚C for 50sec	72˚C for 50sec	72˚C for 50sec
Final extension	72˚C for 5min	72˚C for 5min	72˚C for 5min
Cycles	35	35	35
Hold	4˚C	4˚C	4˚C

Table 2: PCR conditions

3. Result and Discussion

3.1 Isolation of pathogen

Out of 200 vaginal samples screened, *E.coli, Klebsiella pneumonia* were highly positive (Figure 1). Among the isolates, 22% of isolates postive for *E.coli* (22%), *Klebsiella pneumonia* (14%),(Table 3). Among the 45 *E.coli* isolates, 42 were isolated from pragnant and 3 were from non-pragnant womens. (Figure 2). In pregnant and women samples, the prevalence of *Escherichia coli* is significantly high when compare with other isolates. Similarly, Sáez-López et al. (2016) has studied Vaginal *E.coli* (VEC) strains from pragnant womens. Vaginal *E.coli* transmitted through fecal-vaginal-urinary/neonatal route and are responsible for obstetric infections (Sáez-López et al., 2016). This might be due to hormonal changes and a lowering of immunity in pregnant women. But in non-pregnant category, *Escherichia coli*, *Pseudomonas* spp, and *Citrobacter* spp are equally present.

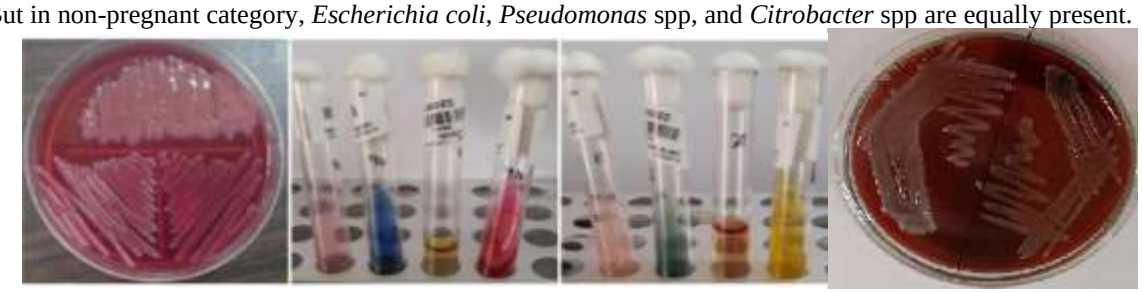

Figure 1: Colonies on MacConkey agar plate and IMViC for 1. *E.coli, Klebsiella pneumonia*

S. No	Bacterial Isolates	Total (n=91/200)
1.	*Escherichia coli*	45 (22%)
2.	*Klebsiella pneumonia*	29 (14%)
3.	*Pseudomonas spp*	9 (4.5%)
4.	*Citrobacter spp*	5 (2.5%)
5.	*Enterobacter spp*	2 (0.1%)
6.	*Proteus spp*	1 (0.5%)

Table 3: Bacterial isolation ratio among pregnant and non-pregnant women

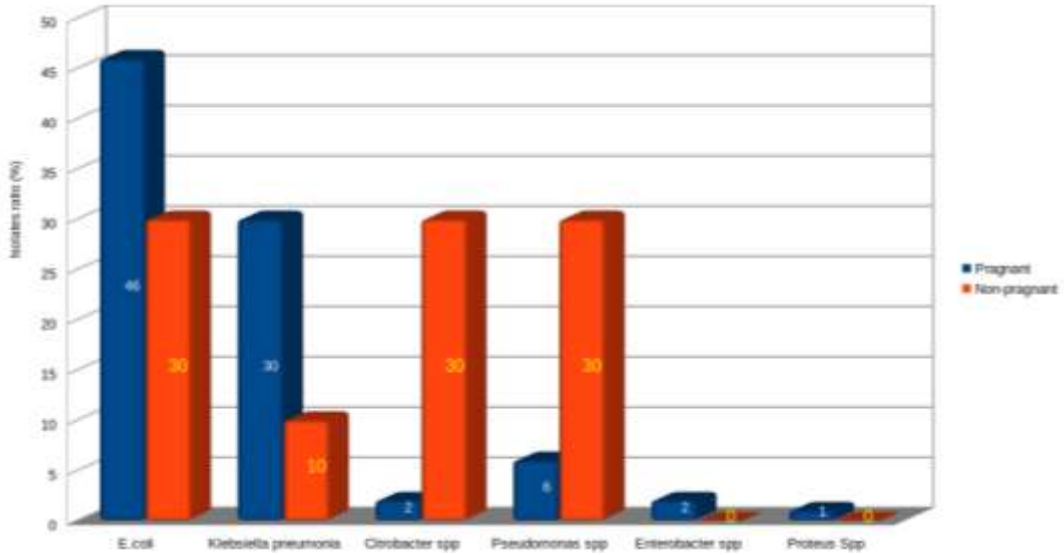

Figure 2: Microbial distribution among vaginal samples

3.2 Antibiotics Sensitivity Test (AST) profile gram negative isolates

The highly screened isolate was subjected ESBL resistance pattern determination (Figure 3). Out of 45 isolates 41 were found to be resistant to cefoxitin (30µg), ceftazidime (30µg), ceftazidime+clavulanic acid (30µg+10µg), aztreonam (30µg), Ciprofloxacin 5µg, Nalidixic acid 30µg. 25 were found to be resistant to cefoxitin 30µg. The prevalence of vaginal Enterobacterales and drug resistance Enterobacterales colonisation of E.coli was more resistant to cefoxitin (10µg) (29.41%), ceftazidime (30µg) (48.23%), aztreonam (30µg) (37.64%), ceftazidime/clavulanic acid (30µg/10µg) (48.23%) imipenem (10g) 7(7.7%), ceftazidime (30g), Piperacillin 90(100%), Cefozolin (30g) 81(90%), Gentamicin (10.g) 12(13.3%), Nalidixic acid (30g) 55(61.1%), ciprofloxacin (5µg) 49(54.4%), Amikacin 14(15.5%). The assay was performed according to CLSI guidelines.

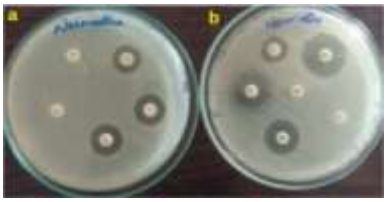

Figure 2: Antimicrobial sensitivity analysis with different antibiotic discs

3.3 MIC determination for gram negative isolatess

The MIC of clinical isolate *Escherichia coli* detected against cefotaxime by using the agar dilution method (hi-media) test. The results showed resistance (MIC value ≥4µg/ml) in 41 isolates. The strain *E.coli* has MIC_{50} of 2 µg/ml MIC_{90} of 512 µg/ml (Figure 4). It indicates the strain is highly resistant to cefotaxime antibiotics. Beta-lactamase genes are highly prevalent in cefotaxime resistant *E.coli* strains (Adegoke et al., 2020; Dehkordi et al., 2020). Based on this obervation, the isolate was taken further for resistance gene pattern determination.

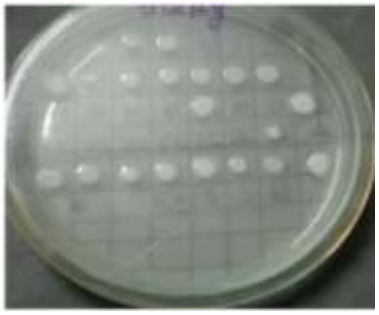

Figure 4: Minimal inhibitory concentration determinations

3.4 Determination of ESBL resistant isolates

CLSI Phenotypic determination or combined disc assay used to screen the Extended-spectrum-lactamase (ESBL) producing isolates from the sample. In this current study, Ceftazidime / Clavulanic acid (CAC-30µg/10µg) produced 22mm and Ceftazidime (CAZ-30µg) has 17mm (Figure 5) The present studies were highly correlated with the done with Uropathogenic E.coli strains (Kukanur et al., 2015).

Figure 5: Combined disc diffusion assay

Epsilometer test or E-strip test uses exponential gradients of antibiotics to determine antimicrobial drug resistance by microbes. In this study, the rectangular strip contained predefined concentration gradient of ceftazidime and clavulanic acid in one end produced inhibitory zone upto 0.38µg/ml against the test isolate (Figure 6). As per the CLSI recommendations, the ratio was above 8 (Humphries et al., 2018). Hence, this confirms the presence of ESBL isolates and this results attributes to the possible presence of inhibitor resistance genes such as TEM, SHV, CTX-M in the ESBL.

Figure 6: E strip test using ceftazidime and ceftazidime+clavulanic acid

3.5 Screening of ESBL resistant genes

Screening the Ambler class A beta lactamases (TEM, SHV, CTX-M) genes were used in the PCR. Taq 2x master mix (5µl), forward and reverse primer (0.5µl), Milli Q water (3µl), and template (1 µl) were used in a final reaction mixture of 10µl. The prevalence of vaginal causing bacteria and drug-resistant bacterial colonisation among pregnant women was examined in this

work. Figure 7, Figure 8 and Figure 9 showed the presence of ESBL resistance genes such as TEM, SHV and CTX-M from the isolates. The isolate *E. coli* (42%) was more predominant, which have been known to be the main causative agents of vaginal infection. Similarly, Wang et al. (2019) has screened ESBL producing E.coli from urinary tract infections.

RESISTANCE DETERMINANTS	NO.OF POSITIVE ISOLATES (N=41)
bla TEM (Figure 7)	27 (65.85%)
bla SHV (Figure 8)	3 (7.31%)
bla CTX-Mgp1 (Figure 9)	27 (65.85%)

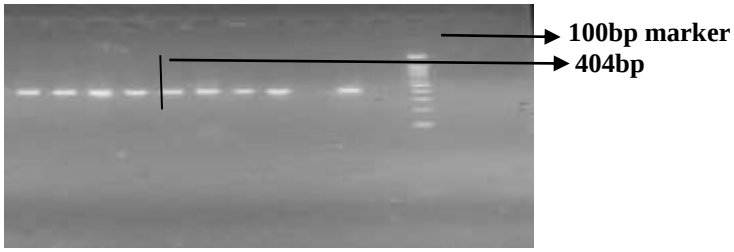

Figure 7: Resistant genes in Agrose gel with TEM gene (standard)

Figure 8: Resistant genes in Agrose gel with SHV gene (standard)

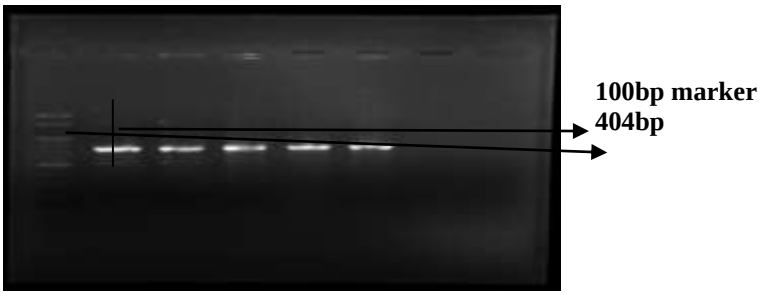

Figure 9: Resistant genes in Agrose gel with CTX-M (standard)

4. Conclusion:

Emergence of drug resistant vaginal *E. coli* has an increasing trend and is a significant clinical challenge, because of limited therapeutic option for this pathogen. Therefore early detection of Extended spectrum beta lactamase producing E. coli is important to restrict their spread in community. In this current study, out of 200 samples collected from vaginosis cases of pragnant and non-pragnant produced 25% postive for *E.coli*. The isolates showed resistance to ESBL antibiotics during combined disc assay and E-strip test. Further, the ESBL resistance genes such as TEM, SHV and CTX-M genes were found in the *E.coli* strain. Hence, we recommend the continuous monitoring of antibiotic susceptibility in E. coli isolates and the resistant isolates to be routinely screened for different kinds of easily detectable virulence factors and beta lactamases to update the characteristics and new types of resistance mechanisms emerging in E. coli.

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