Original Research Article

Prevalence of Vulvovaginal Infections and Species Specific Distribution of Vulvovaginal Candidiasis in Married Women of North India

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ABSTRACT

Abnormal vaginal discharge is a frequent complaint of women of child bearing age seeking gynaecological care. The frequency of different types of vulvovaginal infections (VVI) showed regional variations. Objectives of the present study were to determine the prevalence of different types of VVI and species specific distribution of vulvovaginal candidiasis (VVC) in North India. A total of 200 married women diagnosed by a gynaecologist for VVI were recruited for the present study. Vaginal swabs from these participants were processed for detection of bacterial vaginosis (BV), VVC and trichomoniasis based on European (IUSTI/WHO) guidelines on the management of vaginal discharge (2011). Species specific distribution of VVC was assessed by HiChrome Candida differential agar, Germ tube test as well as Scanning electron microscopy. The findings of the present study indicate that BV was the most prevalent infection (48.5%) followed by VVC (31%) and mixed infections (20.5%). However, no case of trichomoniasis was detected. Candida albicans was found to be the most prevalent species. Out of non-albicans Candida (NAC) species, C. tropicalis was found to be the most prevalent species. Overall distribution of C. albicans and NAC species indicated marginally high prevalence of NAC species (53%) than C. albicans (47%). When VVI were compared between pregnant and non-pregnant subjects, VVC was more prevalent in pregnant females while BV and mixed infections (MI) were more prevalent in non-pregnant females. Furthermore, in pregnant women C. tropicalis (42.8%) was the most prevalent species while in non-pregnant females C. albicans (43.9%) was the most prevalent species. In conclusion, BV was the most prevalent VVI in North India. C. albicans was the single most prevalent species in VVC while among NAC species, C. tropicalis was found to occur at highest frequency.

Keywords

Bacterial vaginosis, Non-albicans Candida species, VVC, HiChrome, SEM, Trichomoniasis
Introduction

Abnormal vaginal discharge is a characteristic feature of vulvovaginal infections (VVI). It occurs in 1-14% of all women of reproductive age throughout the world and its prevalence in India is estimated to be 30% (Thulkar et al., 2010). Most common documented causes of symptomatic vaginal discharge includes bacterial vaginosis (BV), followed by vulvovaginal candidiasis (VVC) and trichomoniasis (Rekha and Jyothi, 2010). Earlier studies have also reported the coexistence of these VVI (French et al., 2004). Epidemiologic data has demonstrated that the prevalence of various reproductive tract infections varies greatly between countries and even between regions within a country. This implies the differences in the characteristics of each pathogen and moreover other biological, behavioral, medical, social, and economic factors (Patel et al., 2003).

BV and VVC involve disturbance in normal vaginal flora which is the main cause of both infections. BV is typified by decrease in the quantity and prevalence of hydrogen peroxide producing lactobacilli and overgrowth of predominantly anaerobic organisms in the vagina while VVC is caused by excessive growth of Candida species which is normally present in small number and is harmless (Sobel, 1988; Holland et al., 2003; Larsson and Forsum, 2005). Untreated chronic BV may result in increased risk of pelvic inflammatory disease (PID), infertility, pre-term birth, premature rupture of membranes, low birth weight, intra-amniotic infections, endometritis, cervical intra-epithelial neoplasia (CIN), post-gynaecological surgery infections and increased risk of sexually transmitted diseases (STD) (Hay et al., 1994; Ralph et al., 1999; Atashili et al., 2008). Chronic VVC can lead to severe vulvovaginal inflammation (Sobel, 1997). Trichomoniasis is a sexually transmitted disease and in untreated cases the risk of spreading other STDs increases (McClelland., 2007).

Some studies have indicated BV to be most prevalent infection, while in some other studies VVC was found to have the highest frequency (Kamara et al., 2000; Gibney et al., 2001; Garcia et al., 2007; Gupta et al., 2009; Shrestha et al., 2011; Chaudhary et al., 2012; Lennox et al., 2013; Sivaranjini et al., 2013; Mobashaeri et al., 2014). C. albicans has been documented to be the major cause of VVC, but the proportion of non-albicans Candida (NAC) species appears to be increasing in last few decades (Stelzner, 1990; Spinillo et al., 1997; Grigoriou et al., 2006; Sobel, 2007; Guzel et al., 2011; Doddaiyah et al., 2014; Hamad et al., 2014; Hedayati et al., 2015). This can be attributed to variety of interventions including single dose treatment, low-dosageazole maintenance regimens and the use of over the counter antymycotics. Studies, in the last decade suggest a NAC prevalence of 10-30% in patients with VVC (Bauters et al., 2002; Corsello et al., 2003; Holland et al., 2003; Vermitsky et al., 2008; Weissenbacher et al., 2009; Zeng et al., 2011). Zeng et al (2011) reported greater percentage of NAC infections than C. albicans infections. Similarly, Grigoriou et al (2006) reported that NAC caused more frequent vaginal soreness and dyspareunia than C. albicans. The predominant NAC species cited in these studies were C. glabrata and C. krusei. A single study reported in India showed that the prevalence of C. tropicalis was even higher than C. albicans.

These differences in occurrence of type of VVI and increasing prevalence of NAC
species over C. albicans show regional variations. In addition, these NAC species were found to be resistant to azoles (Bauters et al., 2002; Singh et al., 2002; Holland et al., 2003; Richter et al., 2005). This sought us to determine the prevalence of causative agent of VVI and species specific distribution of VVC in North India. Furthermore, the distribution of these infections was also compared in pregnant and non-pregnant women. These findings can be helpful in better management of the VVI.

Materials and Methods

Subjects

The present study enrolled 200 patients with symptoms including pruitis, burning, itching, soreness, pelvic pain, vaginal fishy smell, discharge and clinically diagnosed as VVI cases by a gynaecologist. These patients were recruited from Department of Gynaecology and Obstetrics, Bebe Nanki Mother and Child Care Centre, Government Medical College, Amritsar. The subjects with immune deficiencies, using immunosuppressive medications or chemotherapy were excluded from the study. Prior informed consent was taken from all the subjects and the study was approved by institutional ethical committee.

Swab collection and processing

Vaginal discharge samples were collected aseptically from the posterior vaginal fornix using two sterile cotton swabs, speculum and posterior vaginal wall retractor. These vaginal swabs were transferred separately to the labelled vials containing 0.5 ml of physiological saline (0.85% NaCl, pH 7). One vial was placed in ice for the diagnosis of BV and VVC while the other was kept at room temperature for the diagnosis of trichomonomiasis. The material from each vial was divided further into aliquots which were subjected to various diagnostic tests according to European (IUSTI/WHO) guidelines on the management of vaginal discharge (2011).

Identification of Candidiasis

Thick vaginal discharge and vaginal pH of <4.5 were characteristics of candidiasis. The potassium hydroxide (KOH) vaginal preparation was made by mixing equal volume of vaginal solution and 20% KOH in 40% commercially available dimethyl sulphoxide (DMSO). The mixture was incubated for 5 min at 37ºC and was used for microscopic examination of hypae or pseudohypae indicative of Candida infection. The saline suspension of vaginal material from other aliquot was inoculated on saboraud dextrose agar (SDA) (HiMedia, India) and was incubated at 37ºC for 48-72 h. The colonies from SDA were further subtyped by using ready prepared media plates of HiChrome Candida differential agar (HiMedia, India). For this, isolated colonies were streaked on HiChrome agar plates and were incubated at 37ºC in dark for 48 h and the pigmented colonies were examined for species identification. Colonies suggestive of Candida albicans were further confirmed by germ tube test. For this 3-4 small colonies were mixed with 0.5 ml of human serum and incubated at 37ºC for 3 h. Microscopic (Olympus, India) examination was performed in oil immersion (x1000). The strains were maintained in stock media (50% glycerol) at -80ºC pending further use.

Candida species identified with HiChrome agar were further confirmed by Scanning Electron Microscopy (SEM, Zeiss EVO LS 10, Banglore, India). For this colonies from HiChrome agar plates was subcultured on
saboraud dextrose broth at 37°C for 38 h with continuous shaking at 100 rpm in orbital shaker (Scigenics Biotech, India). The broth culture was centrifuged (Eppendorf, India) at 4,000 rpm for 10 min. The pellet thus obtained was washed quickly in five changes of sterile deionized water. The cells were fixed in 2.5% glutaraldehyde (S.d. Chem Ltd., India) at 4ºC for 12 h followed by two washings with sterile deionized water and finally dissolved in sterile distilled water. Twenty µl of this solution was transferred to a round cover slip of 0.1 mm thickness and was air dried for 5 min. Samples were dehydrated serially with various concentrations of ethanol ranging from 50–100%. The cover slips were cemented on aluminium studs with metal glue and coated first with carbon and then silver to a thickness of approximately 30 nm while spinning in vacuum of 10^-5 torr and visualized using scanning electron microscope.

Identification of bacterial vaginosis

Vaginal samples were diagnosed for BV by Amsel’s criteria (Amsel et al., 1983). According to this, BV is present when three of the following four characteristics were detected i.e. thin homogeneous discharge, vaginal pH of >4.5, presence of clue cells and positive whiff test i.e., “fishy” amine odor of vaginal discharge after the addition of 20% KOH in DMSO. Furthermore, differential staining was performed with gram staining kit (K001-1KT) (HiMedia, India) according to manufacturer’s instructions. Briefly, the heat fixed smear was treated with gram’s crystal violet for 1 min followed by gram’s iodine (S013) for 1 min after washing with tap water. The smear was decolorized with gram’s decolorizer (S032) until no further violet color comes off. The slides were washed with water and counter stained with 0.5% w/v saffranin for about 1 min. After washing again with water, the slides were dried and observed under oil immersion objective (x1000). All of the microbial morphotypes were interpreted and quantified by Nugent criteria (Nugent et al., 1991).

Identification of trichomoniasis

The saline solution of vaginal discharge kept at room temperature was further used for identification of trichomoniasis. Thin vaginal discharge with pH > 4.5 and showing positive whiff test were indicative of trichomoniasis. Wet smear (normal saline) test was carried out for microscopic identification of trichomonds motility.

Results and Discussion

The mean age of the studied subjects was 29.5±8.38 years. Out of these, 97% of patients were of pre-menopausal status whereas 3% was of post-menopausal status (Table 1). About 59.5% patients had abnormal vaginal discharge for greater than 5 months. Only 23.5% patients were using one or the other contraceptive methods. In total, 66.5% patients had taken antibiotics during or before two weeks of sample collection and 90% of patients were having random blood sugar (RBS) < 140.

Analysis of vaginal swabs had indicated the presence of BV and VVC while no case of trichomoniais was detected. BV was found to be the most prevalent infection (48.5%) followed by VVC (31%) and MI (20.5%) (Fig. 1). All of the bacterial morphotypes (Fig. 2A-C) were interpreted and quantified by Nugent criteria (Nugent et al., 1991). Candidiasis was indicated by creamy white smooth colonies in SDA culture (Fig. 3i). For identification of Candida species the cases positive for VVC including MI (N=103) were further examined by HiChrome Candida differential agar. The
HiChrome agar showed the presence of five Candida species (Fig. 3ii) i.e., C. albicans, C. tropicalis, C. glabrata, C. krusei and C. dubeligenis. Out of these, C. albicans was the most prevalent species which was further confirmed by germ tube test (Fig. 3iii). Out of NAC species C. tropicalis was the most prevalent species (Fig. 4). These species were further confirmed by SEM based on morphological characteristics (Fig. 5A-E) as described previously (Joshi et al., 1973; Ferreira et al., 2009; Bandara et al., 2010; Thibane et al., 2010; Singhai et al., 2012). Overall distribution of C. albicans and NAC species has indicated marginally high prevalence of NAC species (53%) in comparison to C. albicans (47%).

When only VVC cases (N = 62) were further explored, it was found that 40.3% patients were infected with C. albicans and similar percentage were infected with single NAC species. However, 19.3% cases were found to be infected with mixed species of Candida (Table 2). Similarly, the vaginal sample from patients with MI (N = 41) were further investigated for co-distribution of Candida species with BV (Table 3). Out of these, 48.7% cases were found to have co-infection of C. albicans whereas 46.3% patients were found to be coinfected with one or other single NAC species except C. dubeligenis. Prevalence of NAC species was found to be marginally higher when coinfected with bacteria.

In pregnant and non-pregnant women, the pattern of VVI was similar i.e., BV was the most prevalent infection followed by VVC and MI. However, when pregnant females were compared with non-pregnant females, VVC was more prevalent in pregnant females while BV and MI were more prevalent in non-pregnant females (Fig. 6). When these study groups were compared for only Candida species. C. tropicalis (42.8%) was the most prevalent species in pregnant women, followed by C. albicans (33.3%). However, in non-pregnant women, C. albicans was the most prevalent species (43.9%) while very low prevalence of C. tropicalis (14.6%) was observed (Table 2).

BV, VVC and trichomoniasis have been documented to account for about 90% cases of VVI (Luglio-Agosto, 2005). The present study reported BV to be the most prevalent infection among VVI in North India followed by VVC and MI. This finding is in consonance with various earlier studies which have indicated BV to be the most prevalent cause of VVI (Kamara et al., 2000; Gibney et al., 2001; Garcia et al., 2007; Gupta et al., 2009; Shrestha et al., 2011; Chaudhary et al., 2012; Sivaranjini et al., 2013; Mobashaeri et al., 2014). However, percentage prevalence of BV and VVC in the present study was found to be comparatively higher as reported in earlier studies from India and some other countries (Kamara et al., 2000; Gibney et al., 2001; Garcia et al., 2007; Gupta et al., 2009; Shrestha et al., 2011; Chaudhary et al., 2012; Lennox et al., 2013; Sivaranjini et al., 2013). No case of trichomoniasis was found in the studied group. This finding is in agreement with some earlier reports which have documented very low prevalence of trichomoniasis (Shrestha et al., 2011; Chaudhary et al., 2012). However, some earlier studies in India and some other countries have indicated relatively high prevalence of trichomoniasis (Kamara et al., 2000; Gibney et al., 2001; Garcia et al., 2007; Gupta et al., 2009; Fule et al., 2012). These differences in the prevalence of different types of VVI can be attributed to geographical variations (Patel et al., 2003).

C. albicans was found to be the single most prevalent species in VVC. This finding of the present study was similar to the studies
conducted in Nigeria, USA, Kenya, India, UAE, Iran (Alli et al., 2011; Mintz and Martens, 2013; Nelson et al., 2013; Doddaiah et al., 2014; Hamad et al., 2014; Hedayati et al., 2015). However, this is in contrast to a single study reported in India where C. tropicalis was found to be more prevalent than C. albicans (Sharma and Solanki, 2014). However, in the present study the overall prevalence of NAC species was found to be marginally higher than C. albicans. Among NAC species, C. tropicalis was the most prevalent species followed by C. glabrata, C. krusei and C. dubeligenis. This pattern was not similar when compared to the studies conducted in some other countries including Kenya and USA where C. glabrata was found to be the most prevalent species. However present as well as earlier studies have reported very low prevalence of C. krusei (Leon et al., 2002; Nelson et al., 2013). During the last few years the reports regarding the increased frequency of NAC species in VVC have emerged (Spinillo et al., 1997; Grigoriou et al., 2006; Sobel, 2007; Guzel et al., 2011; Vijaya et al., 2014; Hamad et al., 2014; Hedayati et al., 2015). This may be due to the reason that most of the researchers have not preceded the Candida species differentiation.

### Table 1 Clinical characteristics of patients with vulvovaginal infections

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with VVI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 200</td>
</tr>
<tr>
<td></td>
<td>No. of patients</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
</tr>
<tr>
<td>Pre-menopausal status</td>
<td>194</td>
</tr>
<tr>
<td>Post-menopausal status</td>
<td>6</td>
</tr>
<tr>
<td>Patients having discharge duration for</td>
<td></td>
</tr>
<tr>
<td>1 – 5 months</td>
<td>81</td>
</tr>
<tr>
<td>&gt; 5 months</td>
<td>119</td>
</tr>
<tr>
<td>• 5 months - 1 y</td>
<td>36</td>
</tr>
<tr>
<td>• &gt;1 – 3 y</td>
<td>24</td>
</tr>
<tr>
<td>• &gt;3 – 5 y</td>
<td>5</td>
</tr>
<tr>
<td>• &gt;5 – 10 y</td>
<td>13</td>
</tr>
<tr>
<td>• &gt;10 y</td>
<td>41</td>
</tr>
<tr>
<td>Patients using birth control methods</td>
<td></td>
</tr>
<tr>
<td>OCP</td>
<td>9</td>
</tr>
<tr>
<td>IUCD</td>
<td>11</td>
</tr>
<tr>
<td>Tubectomy</td>
<td>11</td>
</tr>
<tr>
<td>Condom</td>
<td>16</td>
</tr>
<tr>
<td>Patients taken antibiotics in last two weeks</td>
<td></td>
</tr>
<tr>
<td>RBS &gt;140</td>
<td>20</td>
</tr>
<tr>
<td>RBS &lt;140</td>
<td>180</td>
</tr>
</tbody>
</table>

OCP: Oral contraceptive pills, IUCD: Intra uterine contraceptive device.
Fig. 1 Prevalence of vulvovaginal infections

![Pie chart showing prevalence of different infections](image)

- Bacterial Vaginosis: 48.5%
- Vulvovaginal Candidiasis: 31%
- Mixed Infections: 20.5%

Fig. 2 Gram staining of vaginal fluid smears:
- L: Lactobacillus morphotype
- G: Gardnerella morphotype
- N: small Gram-negative rods

(A) Vaginal squamous epithelial cells and only 3+ large gram positive rods (Lactobacillus morphotype), clinical examination was normal.
(B) Mixed vaginal flora including 2+ small gram-positive rods (Gardnerella morphotype) and 4+ large gram-negative rods. No Lactobacillus morphotype was present, clinical diagnosis was BV.
(C) Vaginal squamous epithelial cells with many 4+ large gram negative rods only, clinical diagnosis was BV.
Fig. 3 (i) Saboraud Dextrose Agar (SDA) showing creamy white smooth colonies of Candida. (ii) Colored streaks of Candida colonies on HiCrome Candida differential agar showing differential Candida species Section A: C. albicans; B: C. dubeligenis; C: C. glabrata; D: C. krusei; E: C. tropicalis (iii) Arrow showing germ tube, a characteristic feature of C. albicans

Fig. 4 Overall distribution of VVC in vulvovaginal samples
Fig. 5 Scanning electron micrographs showing various *Candida* species. (A) *C. albicans* colonies with smooth round spores (Length × Breath = 4.507×3.373 µm). A large arrow showing bud scar (B) *C. glabrata* colonies having spherical shape with rough sides and pseudohyphae, smaller than *C. albicans* (2.961×2.130 µm) (C) *C. krusei* colonies having oval to elongated spores with convoluted, rough, irregular and elevated surfaces (3.988×2.275 µm) (D) *C. dubeligenensis* spores having extensively rough convoluted surface (5.049×2.613 µm). (E) *C. tropicalis* colonies showing oval spores with some pseudohyphae. Smallest of all species studied (1.741×622.9 nm). Scale marker 2µm is used to view each sample.
Fig.6 Prevalence of vaginal infections in pregnant and non-pregnant women

![Graph showing prevalence of vaginal infections]

Table.2 Distribution of *Candida* species in vulvovaginal candidiasis patients

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Patients</th>
<th>Pregnant women</th>
<th>Non-pregnant women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 62</td>
<td>N = 21 (33.8)</td>
<td>N = 41 (66.1)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>25 (40.3)</td>
<td>7 (33.3)</td>
<td>18 (43.9)</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>15 (24.1)</td>
<td>9 (42.8)</td>
<td>6 (14.6)</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>7 (11.2)</td>
<td>1 (4.7)</td>
<td>6 (14.6)</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>2 (3.2)</td>
<td>1 (4.7)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td><em>C. dubliniensis</em></td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td><em>C. albicans, C. tropicalis</em></td>
<td>5 (8.0)</td>
<td>2 (9.5)</td>
<td>3 (7.3)</td>
</tr>
<tr>
<td><em>C. albicans, C. glabrata</em></td>
<td>3 (4.8)</td>
<td>1 (4.7)</td>
<td>2 (4.8)</td>
</tr>
<tr>
<td><em>C. albicans, C. glabrata, C. tropicalis</em></td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td><em>C. krusei, C. tropicalis</em></td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td><em>C. dubliniensis, C. glabrata</em></td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td><em>C. glabrata, C. tropicalis</em></td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td>1 (2.4)</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent percentages

Table.3 Distribution of *Candida* species in patients with mixed infections

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Patients</th>
<th>Pregnant women</th>
<th>Non-pregnant women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 41</td>
<td>N = 10 (24.3)</td>
<td>N = 31 (75.6)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>20 (48.7)</td>
<td>5 (50)</td>
<td>15 (48.3)</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>13 (31.7)</td>
<td>2 (20)</td>
<td>11 (35.4)</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>5 (12.1)</td>
<td>2 (20)</td>
<td>3 (9.6)</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>1 (2.4)</td>
<td>1 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. albicans, C. glabrata</em></td>
<td>1 (2.4)</td>
<td>0 (0)</td>
<td>1 (3.2)</td>
</tr>
<tr>
<td><em>C. glabrata, C. krusei</em></td>
<td>1 (2.4)</td>
<td>0 (0)</td>
<td>1 (3.2)</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent percentages
When patients were segregated into pregnant and non-pregnant categories, the overall pattern of VVI was found to be same that is BV was the most prevalent VVI followed by VVC and MI. These findings in case of pregnant women are in consonance with previous studies (Shrestha et al., 2011; Olowe et al., 2014). However, comparison of type of VVI between pregnant and non-pregnant women indicated that VVC was more prevalent in pregnant women than non-pregnant women whereas prevalence of BV and MI was higher in non-pregnant women. Till date no study is available to compare prevalence of VVI in pregnant and non-pregnant women. Furthermore, in case of VVC, C. tropicalis was found to be the most prevalent species in case of pregnant women while C. albicans was the most prevalent species in non-pregnant women. The finding in case of pregnant women was found to be similar to an earlier study conducted in India (Sharma and Solanki, 2014), whereas it is in contrast to the study conducted in Kenya where C. albicans was the most prevalent species in pregnant women followed by C. glabrata, C. tropicalis, C. krusei and C. parapsilosis (Nelson et al., 2013).

The present study indicated BV to be the most common cause of VVI in North India. C. albicans was the most prevalent species and out of non-albicans Candida (NAC) species, C. tropicalis was found to be the most prevalent species. Furthermore, VVC was more prevalent in pregnant females while BV and mixed infections (MI) were more prevalent in non-pregnant females. High prevalence of NAC species in pregnant women indicates their emergence as opportunistic pathogens in immunocompromised conditions. Therefore, screening of pregnant women for differential Candida species can be helpful in better diagnosis of VVI and in providing better antenatal care.

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