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Vaginal Colonization and Susceptibility to Antibiotics of *Enterococci* During Late Pregnancy in Kerman City, Iran

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Abstract

Background: *Enterococci* are a natural part of the genito-intestinal and gastrointestinal normal flora in humans and are widely distributed in the environment and are one of the most important causes of nosocomial infections.
Objectives: The aim of this study was to identify *Enterococcus* spp. from vaginal samples of pregnant women and measure their antibiotic resistance patterns.
Methods: This descriptive study was performed on 602 strains. Vaginal swabs were cultured for *Enterococcus* spp. from pregnant women at 35 - 37 weeks of pregnancy in Kerman city, Iran, during April 2013 to March 2014 or in labor samples transported to the laboratory using Amies transport medium. Swabs were cultivated in Todd Hewitt broth medium and subsequently plated on blood agar plates containing gentamicin and nalidixic acid. Antimicrobial susceptibility testing was performed for *enterococci* by disk diffusion and minimum inhibitory concentrations (MIC).
Results: Vaginal colonization of *Enterococcus* genus was 8.14%. Parameters of age, parity, history of abortion, history of ruptured membranes, vaginal discharge and other vaginal signs (itching and so on) had no influence on vaginal colonization of *Enterococcus* spp. The predominant species were respectively *E. faecalis* 89.8%, *E. faecium* 6.1% and other *Enterococcus* spp. 4.1%. All samples were susceptible to gentamicin and amoxicillin and MIC for gentamicin and amoxicillin was 2 to 8 μg mL^{-1} and 0.5 to 8 μg mL^{-1}, respectively. Resistance to ciprofloxacin was 6.4%, while its MIC range was 8 to 16 μg mL^{-1}.
Conclusions: In this study none of the isolates were resistant to vancomycin, while 20% of isolates with MIC of 4 μg mL^{-1} had intermediate reaction to it. Other demographic parameters had not correlation with vaginal colonization of *enterococci*.

Keywords: Sensitivity, Pregnancy, Iran, *Enterococci*

1. Background

Enterococci are Gram-positive cocci of normal flora in gastrointestinal tract of human and other mammals, although they can be isolated from the oropharynx, female genital tract, and skin of human and animal as well as insects and plants, and can cause opportunistic disease in humans (1-3). *Enterococci*, especially *E. faecalis* are the third factors that cause endocarditis and also postpartum endometritis and abortion (4). *Enterococci* are the most important cause of nosocomial infections, the third main cause of bacteremia and the most frequent cause of infection at surgical intensive care (ICU) units and are the second most common cause of urinary tract infections (UTI) in hospitals. *Enterococci*, as a cause of central nervous system (CNS) infection, are extremely rare (5-7). *Enterococcus faecalis* is amongst the most frequent pathogens found in urinary tract infections (UTIs), which demonstrates that it can incorporate into mature biofilms (8). In the recent years, *enterococci* have been increasingly recognized as important human bacteria causing infections associated with medical devices. Their resistance to most antibacterial agents and their ability to form biofilm has contributed to the increasing incidence of nosocomial enterococcal infections (6).

The *Enterococcus* genus has many species of which *E. faecalis* and *E. faecium* still account for the majority of human infections (9-12). Recent studies have shown a significant increase in the isolation of more unusual species such as *E. durans*, *E. hirae*, *E. gallinarum* and *E. casseliflavus* from clinical samples (12-14).

Aminoglycoside and β-Lactam antibiotics are generally the antibiotic choices for treating serious infections caused by *enterococci* (2). The increasing resistance to antibacterial agents such as penicillins, aminoglycosides

and trimethoprim, and also to glycopeptides such as vancomycin and teicoplanin, creates an increasingly worrisome problem in clinical practice (2, 15). Isolation and species identification of *enterococci* including their resistance to antibacterial agents are reported from Iran (15-18).

Assessment of colonization of pregnant women is important because a high number of widespread nosocomial infections are caused by *enterococci*. Rumyantseva and colleges (19) demonstrated vaginal samples from 100 women with bacterial vaginitis were tested by wet-mount microscopy, Gram stain and quantitative real-time PCR incloud *Enterococcus* spp. were det in 78.3 % cases. Vedmedovska et al. (20) showed that vaginal microflora may be related to diminished fetal growth or low birth weight and suggested a significant role for vaginal organisms in impaired fetal growth, unrelated to preterm birth. These bacteria are important as it causes endocarditis and postpartum endometritis and wound infection in pregnant women.

2. Objectives

The aim of this study was to isolate vaginal flora in pregnant women and identify species and antimicrobial resistance patterns of these bacteria against vancomycin and several other antibiotics in Kerman city, Iran.

3. Methods

3.1. Sample Size

Random sampling was employed and the number of isolates was selected for this study according to the following Equation 1 (P = 0.5; d = 0.1):

$$n \geq \frac{z^2_{\frac{1-\alpha}{2}} P(1-P)}{d^2} = \frac{3.8416 \times 0.5 \times 0.5}{0.12} = 96 \quad (1)$$

3.2. Clinical Isolation of Enterococci

This descriptive study was performed on 602 vaginal samples collected without rupture of membranes from pregnant women attending three major government hospitals including Afzalipoor, Kashani and Kolahdooz hospitals in Kerman city, Iran, during April 2013 to March 2014. These hospitals are general hospitals and include various wards. The medical ethics committee of Kerman University of Medical Sciences approved the study protocol, and all participants provided written consent. The research was in accordance with the current version of the Declaration of Helsinki. The samples were collected at 35 to 37 weeks of gestation or at delivery from women, who agreed to participate in the study. A history sheet was completed according to the information obtained from the patients with the variables shown in Table 1. Women with fever, ruptured membrane or those who received antibiotics during the two weeks prior to sampling were excluded from the study. The isolated *Enterococci* were kept at Cryo Bank (Mast Co England) at -70°C for further analysis (21).

3.3. Identification

The swabs were inoculated into amies transport media (Merck, Germany) and were transported to the laboratory within 24 hours. The swabs were cultured in Todd-Hewitt broth medium for enrichment. Subcultures were made on 5% sheep blood agar plates containing 8 μg mL^{-1} gentamicin and 15μg mL^{-1} nalidixic acid (Mast Diagnostics, Merseyside, UK) to prevent the growth of normal bacterial flora (21, 22). Gram positive, catalase negative, Christie Atkins Munch-Petersen (CAMP) negative and bacitracin resistant isolates were cultured on *enterococci* isolation media (SF broth- Difco Laboratories). For final identification of *enterococci* after 24 to 48 hours of incubation at 37°C (Memmert, Germany), isolates were identified by the following methods (22): Gram staining and bile-aesculin test and growth in presence of 6.5% NaCl. Next, *Enterococcus* spp. was identified by fermentation of carbohydrates such as arabinose and raffinose, pigmentation and arginine decarboxylase (Mast Diagnostics, Merseyside, UK).

3.4. Disk Diffusion Susceptibility Testing

The disk diffusion antimicrobial susceptibility test was done according to the clinical and laboratory standards institute (CLSI) guidelines (23). Antibacterial resistance against vancomycin, ciprofloxacin, amoxicillin and gentamicin were determined using a standard disk diffusion method (Kirby-Bauer) (23). All tests were performed on Muller-Hinton agar (Oxoid Co, Hampshire, UK), and were interpreted after 24 hours of incubation at 37°C. The zone diameter measured around each disk was considered to show complete resistance, intermediate resistance, or sensitivity according to the zone size diameters, and range recommended by the disk manufacturer, which was in accordance with the clinical and laboratory standards institute (CLSI) recommendations.

3.5. Minimum Inhibitory Concentration

We used minimum inhibitory concentrations (MIC) to determine each isolate against four antibiotics: vancomycin, ciprofloxacin, amoxicillin and gentamicin. Stored enterococcal isolates were revived. The inoculums, equivalent to 0.5 McFarland turbidity standards, were

used to streak the surface of the Mueller-Hinton agar. The plates were incubated at 35°C in ambient air for 24 hours. Reading and interpretation of the MICs was done according to the CLSI interpretative standards (21, 23).

3.6. Statistical Analysis

Fisher exact test was used for classified data. $P \leq 0.05$ were considered significant (two-tailed test). Statistical analysis was performed by statistics package for social science (SPSS version 16.0, SPSS Inc., Chicago, IL, USA).

4. Results

From a total of 602 vaginal swabs from pregnant women, 49 (8.14%) isolates were identified as enterococci. Predominant species were respectively, *E. faecalis* 44 (89.8%), *E. faecium* 3 (6.1%), *E. mundtii* 1 (2%) and *E. raffinosus* 1 (2%). There was no significant difference in the rate of vaginal enterococci colonization among pregnant women with respect to age, parity, history of abortion and vaginal sign and symptoms but there was a significant difference in the rate of vaginal *enterococci* colonization among pregnant women with history of membrane rupture (Table 1). All of the 49 *enterococci* isolates were found to be sensitive to amoxicillin, vancomycin and gentamicin. Resistance to ciprofloxacin was found in three (6.4%) of the isolates and the MIC range of the resistant strains was 4 - 8 $\mu g\ mL^{-1}$ (Table 2). All the *enterococcus* species resistance to ciprofloxacin were *E. faecalis* . Results of disk diffusion method and MIC were similar.

5. Discussion

In this study, the carriage rate of *enterococci* in Kerman city, Iran was determined to be 8.14%, which is in agreement with the rates of reports by two Iranian centers (14% and 6.2%) (13, 21). However, the findings of a study by Weinstein and et al. showed rates of 22.9% and 15.6%, which are more than that of our study (24).

In Belgium cultured vaginal and rectal specimens from pregnant women at week 35 to 37 of gestation and vaginal colonization, showed *enterococci* rate of 4.6% that was less than that found by our study (25). In other studies, *Enterococcusfaecalis* was 85% (the most prevalent species), followed by *E. faecium*, which is the most common human pathogenic isolate (10, 25, 26). *Enterococcus faecium* has become increasingly prevalent in hospital-acquired infections (25). All other enterococcal species together constitute less than 5% of enterococcal infections (10, 25). In this study antimicrobial resistance pattern was indicated for *enterococci* with four antibiotics. All isolates were sensitive to amoxicillin, gentamicin and vancomycin. In a review of urinary enterococci by Muratani conducted in Japan, it was shown that resistance to gentamicin in *E. faecalis* and *E. faecium* were 100% and 84.8%, respectively (27).

Kacmaz from Turkey showed that there was a high level of resistance to gentamicin (22% of the isolates) by the disc diffusion method (26). Busani in Italy showed that 41% of *E. faecalis* isolates and 14% of *E. faecium* isolates were resistant to gentamicin (28). Norwegian patients were screened for rectal carriage of Ampicillin-resistant *enterococci* (ARE) and high-level gentamicin-resistant *enterococci* (HLGRE), and a total of 6.9% ARE carriers and 3.3% HLGRE carriers were detected (29). In this study resistance to ciprofloxacin was 6.4% with maximum MIC of 16$\mu g\ mL^{-1}$, and all resistant *enterococci* isolates to ciprofloxacin were *E. faecalis* but all *E. faecium* were sensitive to ciprofloxacin. Busani in Italy showed resistance to ciprofloxacin was 71% and 32%, respectively, for *E. faecium* and *E. faecalis* (28). All isolates were sensitive to vancomycin and 20% of the isolates had intermediate reaction to vancomycin with MIC of 4 $\mu g\ mL^{-1}$. Vancomycin resistance was 20% in a review by Nateghian for *enterococci* in children with acute lymphoblastic leukemia in Iran (18). Kacmaz showed that all isolates were resistant to vancomycin (26). Vancomycin-resistant *enterococcus* (VRE) have been reported among long-term dialysis patients (prevalence = 14%); there were significant associations between VRE and dialysis type (30). No vancomycin resistance was seen in a study of muratani (30). Betriu's study from Spain illustrated that resistance to vancomycin was 5.8% for *E. faecium* and there was no resistance for *E. faecalis* (31). Cheng's study from Hong Kong showed that resistance to vancomycin was 0.32% for *E. faecium* (5). High resistance to vancomycin in other countries is probably due to high usage of this antibiotic in comparison with Kerman city of Iran (32).

There was no correlation between enterococci colonization rate and the demographic factors (Table 1), while there was a correlation between enterococci colonization rate and history of membrane rupture (with OR = 3.18); the risk of *enterococci* infection among pregnant women with a history of rupture is 3.18 times more than that of other groups. The obtained results in relation with *enterococci* are conflicting with the demographic factors. Risk factors like gender, ward, duration of hospitalization and history of prior hospitalization of hospitalized women before childbirth were studied and there was no correlation between these variables and *Enterococci* colonization. However, significant differences in demographic factors were not all the same (17). Identification of risk factors for colonization requires further studies. In this study predominant species were respectively *E. faecalis* 44 (89.8%), *E. faecium* 3 (6.1%), *E. mundtii* 1 (2.05%) and *E. raffinosus* 1 (2.05%),

Table 1. The Relationship Between *Enterococci* Colonization and Demographic Variables in Pregnant Women of Kerman City, Iran[a]

Demographic Variables	Enterococci		P Value
	Patients Tested, No. (%)	Colonization No. (%)	
Age, y			0.11
> 20	87 (14.5)	5 (10.4)	
20 - 24	265 (44)	17 (35.4)	
25 - 35	222 (36.9)	21 (43.75)	
< 30	28 (4.7)	5 (10.4)	
Parity			0.61
0 - 2	397 (65.9)	29 (61.7)	
3 - 5	128 (21.3)	10 (21.3)	
< 5	77 (12.8)	8(17)	
History of abortion			0.14
Yes	91 (15.1)	11 (22.42)	
No	512 (84.9)	38 (77.55)	
Vaginal discharge			0.3
Yes	150 (24.9)	9 (18.37)	
No	452 (75.1)	40 (81.63)	
Vaginal sign (itching)			0.14
Yes	126 (20.9)	6 (12.25)	
No	476 (79.1)	43 (87.75)	
History of ruptured membrane			0.01
Yes	152 (25.3)	5 (10.2)	
No	450 (74.8)	44 (89.8)	

[a] OR = 3.18, *enterococci* had an infection chance of 3.18 more than other groups in pregnant women with a history of ruptured membrane.

Table 2. Antimicrobial Susceptibility of *Enterococci* Isolates (n = 49) From Pregnant Women of Kerman City, Iran

Antibacterial Agent	MIC Range, $\mu g\ mL^{-1}$	MIC 90, $\mu g\ mL^{-1}$	Resistance (%)
Amoxicillin	$\leq$ 0.03 - 1	$\leq$ 0.03	0
Gentamicin	$\leq$ 2 - 4	$\leq$ 2	0
Vancomycin	$\leq$ 0.03 - 4	$\leq$ 4	0
Ciprofloxacin	$\leq$ 0.03 - 8	$\leq$ 4	6.4

which is in accordance with the findings of other conducted studies worldwide.

There may be more risk factors for enterococci colonization, which are still unknown and further studies with more samples are required to determine the relationship between these risk factors and enterococci colonization in pregnant women.

5.1. Conclusion

Due to controlled use of antibiotics in this area of Iran, there was not high rate colonization of *enterococci*. These bacteria are the most important causes of nosocomial infections. Resistance of *enterococci* has increased towards antibacterial agents such as trimethoprim, penicillins and aminoglycosides, and also to glycopeptides such as teicoplanin and vancomycin, which is an increasingly worrisome problem in clinical practice. In order to control this problem, all strains should be tested for antibiotic suscep-

tibility before use. In this study two hundred and one samples taken from the total sample (803) were lost during culture. Our strategy for solving this problem was to reduce the time of freezing. The positive point of this research was the large sample size. It is suggested that research and molecular typing of isolates should be checked.

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Footnotes

Authors' Contribution: Ehsan Ghasemi developed the original idea and the protocol, study concept and design, acquisition of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, administrative, technical and material support and study supervision. Shahla Mansouri developed the original idea and the protocol, study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, administrative, technical and material support, study supervision. Naser Shahabinejad participated in the acquisition of data.

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