

Genetic Determination of Trimethoprim Resistance in Female Patients Suffering from Vaginal Infections

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Abstract

During the study period, 45 samples were taken from female patients who complaining from vaginal infections. Cultural and biochemical characterization were performed followed by using api-20 E system for more accurate diagnosis. Results showed that out of the total number of samples, 36 isolates belonged to Enterobacteriaceae. In order to determine the susceptibility of the isolate to trimethoprim, disk diffusion method was used where results showed that 10 of 16 *Escherichia coli* isolates were resistant, all five *Proteus mirabilis* isolates were also resistant, while 2 of 5 isolates of *Enterobacter cloacae* were resistant, and 7 isolates of *Klebsiella pneumoniae* were susceptible, 3 were resistant and 1 isolate was intermediate.

Genetic detection of distribution of *dfrA7* gene among isolates were performed by polymerase chain reaction technique. Most *E.coli* possessed this gene, only 2 isolates of *P. mirabilis*, 3 of *E.cloacae*, and *K.pneumoniae* with one isolate.

Key words: trimethoprim, vaginal infections, *dfrA7*, PCR

الخلاصة

خلال فترة الدراسة تم اخذ 45 عينة مهبلية من اناث يشتكين من التهابات مهبلية. شخّصت العينات تشخيصاً زرعياً وكيميائياً متبوعاً باستخدام نظام api-20E لتشخيص أكثر دقة. اظهرت النتائج ان 36 عينة كانت عائدة للعائلة المعوية. استخدمت طريقة الانتشار بالاقراص لتحديد مدى حساسية العزلات للتراميثوبريم. اظهرت النتائج ان 10 عزلات من 16 عذلة *E. coli* كانت مقاومة، وجميع عزلات *P. mirabilis* الخمسة كانت مقاومة ايضاً، 2 من 5 عزلات من *Enterobacter cloacae* كانت مقاومة و بالنسبة لبكتريا *Klebsiella pneumoniae* فكان 7 منها حساسة و 3 مقاومة و 1 متوسطة. استخدمت طريقة PCR لغرض التحري جينياً عن مدى انتشار جين *dfrA7* بين العزلات، اظهرت النتائج ان اغلب عزلات *E. Coli* تمتلك هذا الجين، و اثنان من *P. mirabilis* وثلاث من *E. cloacae* و *K. pneumoniae* بعزلة واحدة فقط.

الكلمات المفتاحية: ترايميثوبريم، التهابات مهبلية، جين *dfrA7*، تشخيص جيني

Introduction

The normal microbiota of vagina play a pivotal role in protecting it from invading pathogens, including those that cause urinary tract infections and sexually transmitted diseases (Boris *et al.*, 1998.). Any disruption in the normal vaginal ecosystem changes the structure of microflora of the healthy vagina, altering vaginal pH and predisposing to lower tract infections such as vaginitis. A balanced vaginal ecosystem depends on *Lactobacillus spp.*, which are essential for maintaining the vaginal pH at 4.5 and assisting host defenses to inhibit over growth of other pathogenic bacteria (obligate anaerobes) that can lead to infection. The infection of vagina (vaginitis) can be caused by various agents like bacteria, *Candida*, and *Trichomonas vaginitis*, and viruses (Kent, 1991).

Although *E.coli* can be counted within the vaginal normal flora of pregnant as well as non pregnant women it responsible for symptomatic infection of it. (Gillespie and Hawkey 2006). The year 1988 had witnessed the Nobel Prize for Medicine to be awarded to Hitchings and Elion for their discovery of the diaminopyrimidines, that includes trimethoprim (TMP). The biological activity of trimethoprim is attributed to Schiff base (-CH=N-). These are a group of potent antibiotics that exhibit a high degree of specificity for bacterial dihydrofolate reductase due to structural differences between the bacterial and mammalian enzymes. (Hitchings, 1989).

Trimethoprim is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system(WHO Model List of Essential Medicines". (World Health Organization October 2013).

Trimethoprim resistance in clinically significant Gram-negative bacteria is usually caused by horizontally transferable resistance genes (*dfr*) genes coding for alternative resistant dihydrofolatereductases. Most of such genes can be found as gene cassettes carried by integrons forming parts of transposons, which mediate wide spread dissemination of trimethoprim resistance(Grape *et al.* , 2007)

Since our knowledge of resistance to trimethoprim in developing countries in general and our country in particular is not extensive. Additionally Some reports indicate that the prevalence of resistance in enterobacterial pathogens isolated in these countries is very high (33–96%) compared to isolates from developed countries (7–24%). (Frank *et al.*,2007).

Besides the rarity of molecular studies on this drug in Iraq, this research was devote to :

- Isolation and identification of bacterial agents causing symptoms of vaginitis in women visited some of Al-Najaf hospitals
- Antimicrobial susceptibility of bacterial isolated for trimethoprim.
- Genetic detection of *dfrA7* gene in bacterial isolates.

Methods

1-Sample Collection

From the end of July 2014 to November 2014, 45 samples were collected from female patients suffering from vaginal infection who visited some of Al-Najaf province hospitals. The samples then submitted to routine bacterial diagnosis procedures, comprising the cultural, biochemical and further more by using diagnostic kits (api- 20E).

2- Trimethoprim susceptibility test

All isolates performed identification to susceptibility testing to trimmethoprim (trimmethoprim disks 5 µg by Himedia) according to modified disc-diffusion method (Kirby-Bauer) (Bauer *et al.*, 1966). Results were interpreted according to (CLSI,2013)

3-Molecular detection of Trimethoprim Resistance:

a. Isolation of Genomic DNA.

Genomic DNA was isolated by using prepared kit (geneaid, Taiwan), and according to the instructions of manufacturing company.

b. PCR Technique

Polymerase chain reaction assays were carried out in a 25 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set (Biocorp, Quebec, Canada) depending on their reference procedure, (Frank *et al.*, 2007) for *dfrA 7* gene.

4- PCR Product Analysis

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products were loaded to the agarose gel wells: 5µl from single product to single well in known sequence, followed by 100 bp ladder (Bioneer Korea) to one of the wells in each row. The electric current was performed at 60 volt for 1.5 hour. The electrophoresis result was detected by using gel documentation. The base pair of DNA bands were measured according to the ladder. The positive results were distinguished when there was DNA band equal to the target product size. Finally, the gel was photographed using gel documentation saving picture.

Results and Discussion

1-Isolation and Identification

This study aimed at recover various types of Enterobacteriaceae species from female patients visited some of Al-Najaf hospitals suffering from vaginitis. High vaginal swabs were taken from the patients to get best results. During the study, 45 samples were collected, comprised female age range between (21-37) years with a mean (28.1 ± 4.68). Positive culture for Enterobacteriaceae bacterial diagnosis was 36 isolate. Those were comprising 16 *Escherichia coli*, 5 *Enterobacter cloacae*, 5 *Proteus mirabilis*, and 10 *Klebsiella pneumoniae* as in the table below

Table (4 -1) Distribution of Samples according to the species isolated

Bacterial species	No	Percent%
<i>Escherichia coli</i>	16	44.4
<i>Enterobacter cloacae</i>	5	13.8
<i>Proteus mirabilis</i>	5	13.8
<i>Klebsiella pneumonia</i>	10	28
Total	36	100

Results were different from that of Sharief (1998), where she found that *klebsiella* members were highest in isolation number among Enterobacteriaceae with 26 isolates followed by *E.coli* with 8 isolates and finally *Proteus* with only 4 isolates.

Studies showed that *E.coli* was the first in isolation rate among Enterobacteriaceae where they revealed that the colonization of vagina is attributed to two types of factors both exogenous and endogenous; the first represented by that some women as compared with healthy controls had history of recurrent urinary tract infections, which is due to possession of *E. coli* increased rates of attachments to the epithelial and uroepithelial cells, besides its ability to colonize the vagina in periods of recurrence. the second; the endogenous factors are represented by the deficiency of antibody in cervicovagina of which on the other hand could cause recurrence in urinary tract infections. Some women could develop UTI synochronizedly with colonization of their vagina with *E. coli* but the later may be transient and not obvious. (Hooton and Stamm, 1996)

The virulence factors involved in infection related with the female reproductive tract are different from that of the intestinal tract which is highly related with ability to adherence (expression of some adhesins) (Cook *et al.*, 2001)

Modern methods had been used for more accurate diagnosis of vaginal microbiota., which included the molecular characterization techniques like, sequencing, PCR, DNA fingerprinting, or DNA hybridization (Janneke *et al.*, 2014)

(4.2) Trimethoprim Susceptibility Testing

In order to determine the response extent the isolates to the trimethoprim the disk diffusion method was used with concentration (5) µg.(table 4-2)

Table (4 -2) Trimethoprim Susceptibility Testing of the isolates

Bacteria	Susceptible	resistant	Moderate	Total
<i>Escherichia coli</i>	2	14	0	16
<i>Enterobacter cloacae</i>	2	3	0	5
<i>Proteus mirabilis</i>	0	5	0	5
<i>Klebsiella pneumonia</i>	7	2	1	10
Total	11	24	1	36

Results showed, out of 36 isolates 24 isolate were resistant, 11 susceptible, and 1 isolate was moderate. Most *E. coli* isolate were resistant with 24 isolates. As comparing with former studies we observe that this antibiotic had great effectiveness against *E. coli* as well as other members of Enterobacteriaceae (Haase *et al.*., 1984). Other study showed that from more than 600 *Escherichia coli* isolates, about 400 isolates were resistant to trimethoprim with gradual decrease in resistance frequency along twenty two years comprised in that study (Yu *et al.*.,2004). Similarly a study by Labar *et al.*,(2012) revealed that among various types of antimicrobial agents used their were a sequential raise in resistance with prominent exception of studied agent(trimethoprim) that maintained its resistance rate as high as 45percent .

(4.3) Molecular detection of Trimethoprim Resistance Gene

In order to detect the possession of the isolated bacteria for genes responsible for resistance to trimethoprim, two oligonucleotide sequence were used, results showed that 20 isolates had the gene *dfrA7*, while 16 isolates were negative.

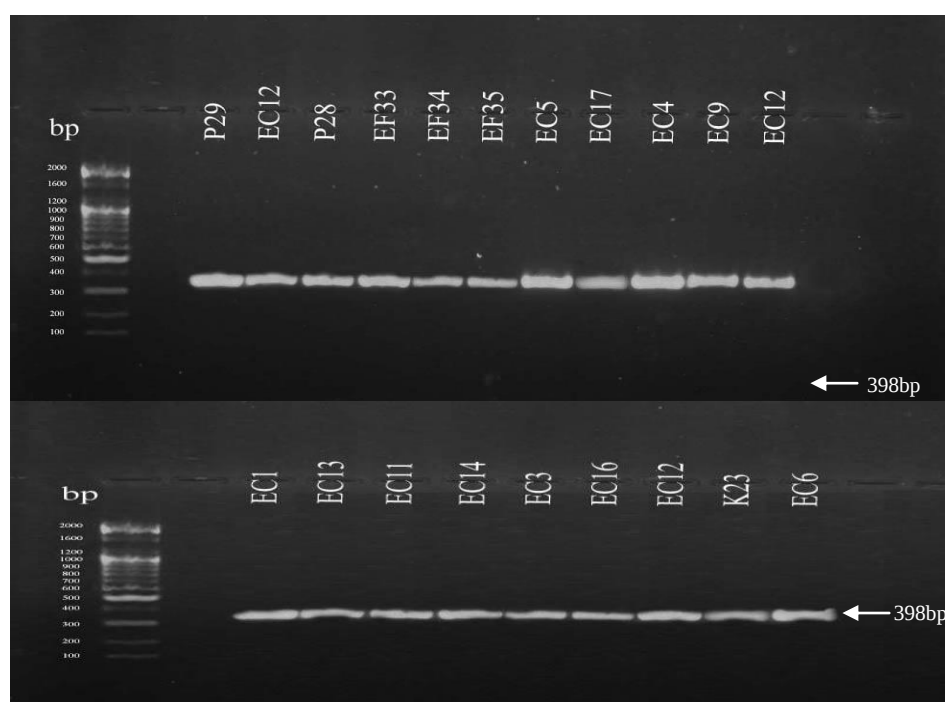


Figure (4-1): Ethidium bromide-stained agarose gel of PCR amplified products from extracted enterobacteriaceae DNA amplified with primers *dfrA7* F and *dfrA7*R. The Lane on the left, DNA molecular size marker (ladder).

From the results we noted that *Escherichia coli* is in the forefront of possession of *dfrA7* gene among studied bacteria. Correspondingly these isolates were multidrug resistant to various types of antimicrobial agents, besides a significant fraction of them had the ability to resist trimethoprim. The most frequently involved mechanism of this agent resistance represented by presence of the gene *dfr* and its ability to disseminate by plasmids, transposons among different isolates. The gene *dfr* have various types which may reach 17 kind present in Gram negative bacteria such as *dfrA5*, *dfrA7* and *dfrB3* and could be present on integron class-1 (Yu *et al.*,2004)

The possession of *Escherichia coli* of more than 30 known *dfr* genes that code for resistant variants, mostly falling into the category of *dfrA* gene, more specifically *dfrA7* which occur by mobile dissemination through integrons that show regional dissemination pattern made this bacteria the most frequently resistant to trimethoprim. (Labar *et al*.,2012)

High resistance to trimethoprim may be due to mutations in the intrinsic *dfr* gene in some pathogens including *Escherichia coli*. While the low level of resistance to this agent indirectly because of loss of capacity of methylation of deoxyuridylic acid to thymidylic acid mutationally which force bacteria to get thymidine from external sources. (Skold,2001)

Regarding other isolates, negative results do not necessarily mean absence of the resistance gene, but it may be attributed to presence of other variant of the *dfrA7* allele or simply resistance is due to other *dfrA* alleles or even *dfr* genes.

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