Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Investigation of phylogroups and some virulence traits among cervico-vaginal *Escherichia coli* (CVEC) isolated for female in Hilla City, Iraq

Marwa Mohammed Al-Khaqani, Mourouge Saadi Alwash, Hussein Oleiwi Al-Dahmoshi*

University of Babylon, College of Science-Biology Department, Iraq.
Email: dr.dahmoshi@yahoo.com

Received 8 September 2016; Received in revised form 23 October 2016; Accepted 9 November 2016

ABSTRACT

Aims: This study aims to investigate the phylogroups, antibiotics susceptibility and biofilm formation among CVEC isolated from female with bacterial vaginosis.
Methodology and results: High vaginal swab from girl with age (18-60 years) were collected and cultured on MacConkey agar, EMB agar and UTI chromogenic medium to recover CVEC and only the confirmed *Escherichia coli* will pass through rest of the assays like phylogrouping (by PCR), antibiotics susceptibility test and biofilm formation. The results revealed that only 32 (20.38%) of CVEC were recovered and among them only 3 (9.375%) of CVEC belong to intestinal subgroup A1 and the rest 29 (90.625%) assigned to extraintestinal phylogenetic group B2. CVEC isolates belong to B1 and D groups not reported. Antibiotics resistance results shown that, 32 (100%) for cefazolin, cephalothin, cefoxitin and metronidazole, 31 (96.9%) for erythromycin, 24 (75%) for fosfomycin, 20 (62.5%) for cefotaxime, 16 (50%) for ceftazidime, 14 (43.75%) for cefepeim, (28.1%) for aztreonam, 7 (21.9%) for streptomycin, 6 (18.75%) for meropenem, 5 (15.6%) for both imipenem and gentamicin, 2 (6.25%) for both ciprofloxacin and norfloxacin, amikacin 1 (3.1%) and no resistance stated for nitrofurantion (0.00%). TCP methods results display that 12 (37.5%) of CVEC were biofilm former while 20 (62.5%) were non biofilm former.
Conclusion, significance and impact of study: This study concluded that, most of the CVEC belong to highly virulent phylogroup B2 and have the ability to resist multiple antibiotics and the ciprofloxacin, norfloxacin, amikacin and nitrofurantion still the best choice for treatment and CVEC have the ability to form biofilm which make the infection ruthless and hard to cure.

Keywords: CVEC, phylogrouping, chuA, yjaA, biofilm

INTRODUCTION

Bacterial vaginosis (BV) is the most common vaginal infections among women in reproductive age. It is a condition of vaginal flora imbalance, in which the typically plentiful H_2O_2 producing lactobacilli are scarce and other bacteria such as *E. coli* is abundant (Hemalatha *et al.*, 2013). BV has been implicated as a risk factor for adverse pregnancy outcomes such as preterm birth, recurrent abortions, post-abortal sepsis, early miscarriages and still births (Africa *et al.*, 2014).

Escherichia coli members that cause infections other than intestinal called extraintestinal pathogenic *E. coli* (ExPEC). ExPEC include those cause urinary tract infections (UPEC), cervix and vagina infections (CVEC), meningitis and sepsis meningitis-(MNEC) (Russo and Johnson, 2000). All of them according to site of infection regards ExPEC but may be from intestine origin (intestinal pathogenic *E. coli* called InPEC) and reach to the extraintestinal regions like those ascended from the anal region of female to vagina due to proximity of the anus to the vagina (Heinemann and Reid, 2005).

Discrimination between InPEC and ExPEC it is very important and can predict the virulence factors owned by CVEC. Characterization the phylogroups using PCR were established using the genetic markers chuA, yjaA and the DNA fragment TspE4.C2 (Clermont *et al.*, 2000). Phylogenetic analysis has shown that *E. coli strains* can be assigned to one of the main phylogroups (A, B1, B2, and D). Intestinal pathogenic *E. coli* (InPEC) include group A (A0 and A1 subgroups) and group B1 (Only B1 subgroup). Extraintestinal pathogenic *E. coli* (ExPEC) include group B2 ($B2_2$ and $B2_3$ subgroups) and group D (D1 and D2 subgroups) (Rodriguez-Siek *et al.*, 2005; Escobar-Pramo *et al.*, 2006).

Studying the phylogroups and virulence factors of *E. coli* isolated from female reproductive tract infection (RTI) were carried out and found that CVEC have unique properties that may enhance their virulence. These properties are similar to those associated with other extraintestinal pathogenic *E. coli*, where most of them were derived from phylogenetic group B2 and D and

*Corresponding author

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

possess numerous virulence factors such as adhesins, toxins, siderophores and polysaccharide coatings. Studies from worldwide have reported isolation of drug resistant *E. coli* among vaginal isolates of pregnant women. Transmission of these resistant strains to the neonate can prove fatal in whom early detection was challenging and treatment options are limited. Antibiotics resistance emerged and rapidly propagated worldwide and threatening the efficacy of antibiotics (Devi *et al.*, 2014).

Generally *E. coli* have four main resistance mechanisms: (i) direct enzymatic antibiotic of the active antibiotic molecule and this is a prominent resistance mechanism toward β-Lactam, aminoglycosides and fluoroquinolones and metronidazole; (ii) Target modification and this noticeable for aminoglycoside, fluoroquinolones and fosfomycin resistance (Pumbwe *et al.*, 2008); (iii) Efflux pumps and outer membrane (OM) impermeability without modification of the antibiotic itself and this is clear resistance mechanism β-Lactam, aminoglycosides and fluoroquinolones and nitrofurantion or (iv) Target bypass like those guaranteed resistance for trimethoprim-sulfamethoxazole (Wong *et al.*, 2015; Ho *et al.*, 2016).

Biofilm formation is considered as a marker of clinically relevant infection and persistence of bacterial biofilms in the human body is a major cause of recurrent or chronic infections (Murugan *et al.*, 2011). It mediates interaction between bacteria and host tissue through adhesion. Biofilms are not only resistant to antibiotics but also to a variety of disinfectants which emphasizes that their characterization is an important aspect of infection control (Mathur *et al.*, 2006). Biofilm formation have a role in persistence of bacterial vaginosis and provide an anatomic haven that protect bacteria from the effects of antibiotics and perpetuate the bacterial vaginosis and rendering them hard to cure (Swidsinski *et al.*, 2007; Fakruddin *et al.*, 2014). This study aims to investigate the phylogroups, antibiotics susceptibility and biofilm formation among CVEC isolated from female with bacterial vaginosis.

MATERIALS AND METHODS

Sample collection

From October 2015 to January 2016, One hundred fifty seven (157) high vaginal swabs were collected from women suffering from vaginitis with age (18-60 years) who visit the gynecology consultant of Babylon maternity and children hospital, and Al-Qassim hospital. Immediate checking of color and pH of vaginal secretion were performed at the clinic. The swabs were inserted into the posterior fornix, upper part of the vagina and rotated there before withdrawing them. A vaginal speculum was also used to provide a clear sight of the cervix and the swab was rubbed in and around the introitus of the cervix and withdrawn without any possible contamination of the vaginal wall.

Microbiological study

All swabs were placed in tubes containing Brain heart infusion broth (BHIB) used for transportation of specimens to laboratory. The swabs were inoculated on MacConkey agar (to check the ability of bacterial isolates for lactose fermentation (pink colony) (Himedia/India) and then the Gram-negative, oxidase negative bacilli transferred to UTI chromogenic medium (Condalab/Spain) to check the pink colony and Eosin methylene blue agar (Himedia/India) green metallic sheen) to confirm *E. coli* (cervico-vaginal *E. coli*). All plates were incubated aerobically at 37 °C for 24 h.

DNA extraction

The pure CVEC isolates we inoculated in LB broth (Condalab/Spain) at 37 °C for 18 h. Harvesting and washing with PBS (Condalab/Spain) for three times and then following the protocols of FavorPrep Genomic DNA Mini Kit (Blood/Cultured Cell) (Favorgen/Taiwan). The extracted DNA checked using agarose gel electrophoresis (0.7% in TBE buffer) (Condalab/Spain) and then visualized using and gel documentation (Vilber/France).

Phylogrouping study

Polymerase chain reaction were used to investigate the phylogroups using three markers: chuA, yjaA and TspE4C2 using 20 µL reaction mix (IntronBio/Korea). The thermocycler (Techno/UK) condition were initial denaturation at 95 °C for 4 min; 30 cycles of (denaturation at 94 °C for 30 sec), (annealing at 59 °C for 30 sec), (extension at 72 °C for 30 sec) and final extension at 72 °C for 5 min. Agarose gel electrophoresis (1.5% in TBE buffer) and gel documentation (Vilber/France) were used to visualized and document the PCR products. The amplicon sizes were 279 bp for chuA, 211 bp for yjaA and 152 bp for TspE4C2 were recorder using 100 bp ladder (IntronBio/Korea).

Antibiotics susceptibility test

The *in vitro* susceptibility of *E. coli* isolates to *18 antimicrobial agents* were determined via disk diffusion method according to Clinical and Laboratory Standards Institute instructions (CLSI, 2016). Activation of isolates were performed using nutrient broth for 18 h at 37 °C and the growth was adjusted to 0.5 McFarland's standard (10^8 CFU/mL) and then spread on Muller Hinton agar (MHA) with a sterile cotton swab. Antibiotic disks were placed onto MHA, gently pressed down to ensure complete contact with the agar inoculated with bacteria and then incubated for 24 h at 37 °C and then inhibition zone diameter in millimeters (mm) was recorded. Interpretation of results as a sensitive or resist were achieved according to CLSI (2016).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Biofilm formation assay

Tissue culture plate method (TCP) assay (also called semi quantitative microtiter plate test (biofilm assay) described by Christensen *et al*., (1985) was most widely used and was considered as standard test for detection of biofilm formation as follow: Isolates from fresh agar plates were inoculated in TSB containing 1% glucose and incubated for 18 h at 37 °C and then diluted 1:100 with fresh TSB. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates wells were filled with 150 µL aliquots of the diluted cultures and only broth served as control to check non-specific binding of media. Each isolate was inoculated in triplicate. The tissue culture plates were incubated for 24 h at 37 °C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed by placing in oven at 37 °C for 30 min. All wells stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. A 150 µL of acetone/ethanol (20:80 v/v) mixture were added to dissolve bounded crystal violet. The optical density (OD) at 630 nm were recorded and the results were interpreted according to Stepanovic *et al*. (2007) as follow:

Non-adherent when	OD ≤ ODc
Weakly adherent when	ODc < OD ≤ 2 × ODc
Moderately adherent when	2 × ODc < OD ≤ 4 × ODc
Strongly adherent when	4 × ODc < OD

OD of cut-off (ODc)= Mean of OD of negative control + 3× Std. Deviation of OD of negative control.

Biosafety and hazard material disposing

Biosafety aspects followed during the work include disposing of all swabs, petri dishes and all contaminated supplies by autoclaving and then incineration. All benches cleaned with alcohol before and after the work. SimplySafe (Eurx/Poland) were used instead of ethidium bromide.

RESULTS

Phylogroups of CVEC isolates

Thirty two (20.38%) Cervico-vaginal *E. coli* (CVEC) isolates were recovered from 157 female suffering from vaginitis. All CVEC isolates were subjected to phylogrouping by PCR according to Clermont *et al*. (2000) using three markers: chuA, yjaA and TspE4C2. According to the presence and absence of each gene, the CVEC isolate will assigned to one of four phylogroup, group A and B1 (intestinal groups); B2 and D (extraintestinal groups). Figures 1, 2 and 3 show 1.5% Agarose gel electrophoresis for *chuA* amplicon (279 bp), *yjaA* amplicon (211 bp) and TspE4C2 amplicon (152 bp) respectively. The results revealed that, only 3 (9.375%) of CVEC belong to intestinal subgroup A1. The rest 29 (90.625%) of CVEC isolates assigned to extraintestinal phylogenetic group B2. CVEC isolates belong to B1 and D groups not reported (Table 1).

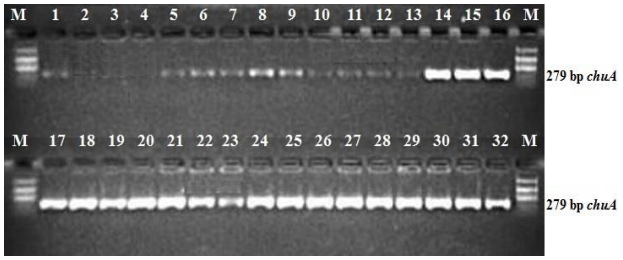

Figure 1: 1.5% Agarose gel electrophoresis for *chuA* amplicon (279 bp). Lane M 100 bp DNA marker, lane 1-32 isolate of CVEC. All isolates were positive while isolate no. 2, 3, 4 were negative.

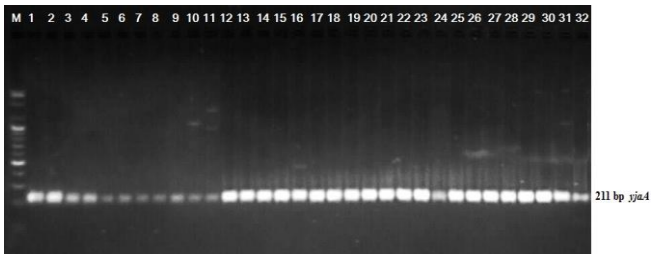

Figure 2: 1.5% Agarose gel electrophoresis for *yjaA* amplicon (211 bp). Lane M 100 bp DNA marker, lane 1-32 isolate of CVEC. All isolates were positive.

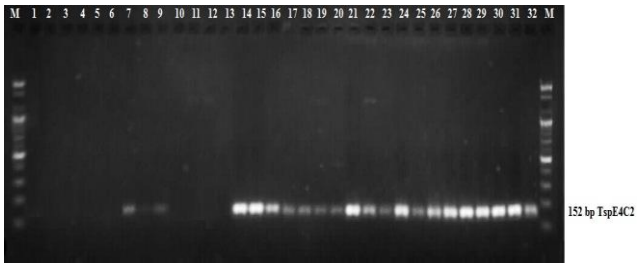

Figure 3: 1.5% Agarose gel electrophoresis for TspE4C2 amplicon (152 bp). Lane M 100 bp DNA marker, lane 1-32 isolate of CVEC. All isolates were positive except 1-6, 8, 10-13.

Table 1: Distribution of CVEC among phylogenetic subgroups.

Phylogenic group	*chuA/ yjaA*/TspE4cC2	No. (%)
Group A	-/-/- or - /+/-	3 (9.375)
Group B1	-/-/+	0 (0.000)
Group B2	+/+/- or +/+/+	29 (90.625)
Group D	+/-/- or +/-/+	0 (0.00)

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Antibiotics susceptibility among CVEC isolates

All tested antibiotics were selected according to CLSI (2016) guidelines. Kirby-Bauer Disc diffusion method were used to show the antibiotic susceptibility of CVEC isolates. Eighteen antibiotics were used (9 antibiotics were cell wall synthesis inhibitor), (4 antibiotics were protein synthesis inhibitors) and (5 antibiotics were DNA synthesis inhibitors). The resistance to antibiotics that inhibit cell wall synthesis the results were 32 (100%) for cefazolin, cephalothin and cefoxitin, 20 (62.5%) for cefotaxime, 16 (50%) for ceftazidime, 14 (43.75%) for cefepeim, 9 (28.1%) for aztreonam, 6 (18.75%) for meropenem, 5 (15.6%) for imipenem Figure 4, and fosfomycin, 24 (75%) (Figure 5).

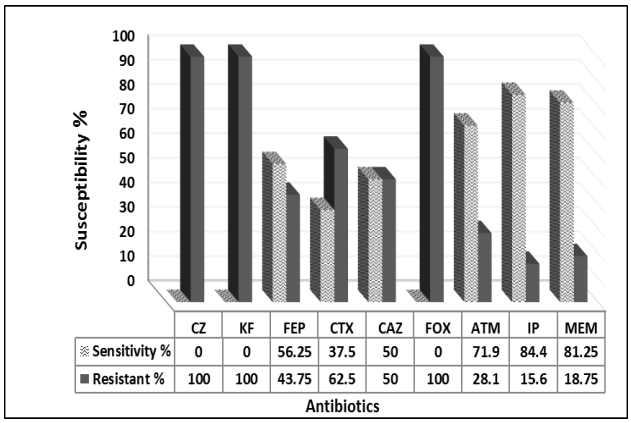

Figure 4: Antibiotics resistance among CVEC for Cephems, Monobactams and Carbapenems. Cefazolin (CZ); cephalothin (KF); cefepeim (FEP); cefotaxime (CTX); ceftazidime (CAZ); cefoxitin (FOX); aztreonam (ATM); imipenem (IP); meropenem (MEM).

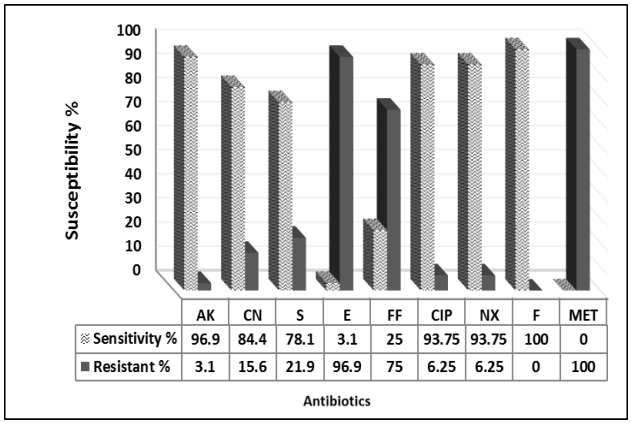

Figure 5: Antibiotics resistance among CVEC for Aminoglycosides, Fosfomycins, Fluoroquinolones, Nitrofurans and Nitroimidazoles. Amikacin (AK); gentamicin (CN); streptomycin (S); erythromycin (E); fosfomycin (FF); ciprofloxacin (CIP); norfloxacin (NX); Nitrofurantion (F); metronidazole (MET).

The resistance to protein synthesis inhibiting antibiotics revealed high resistance to erythromycin 31 (96.9%) and less resistance to streptomycin 7 (21.9%), gentamicin 5 (15.6%) and amikacin 1 (3.1%). The resistance to antibiotics that inhibit DNA synthesis were high. For metronidazole 32 (100%), 2 (6.25%) for both ciprofloxacin and norfloxacin. All isolates were sensitive for nitrofurantion.

Biofilm formation among CVEC

The ability of CVEC to form biofilm were evaluated using tissue culture plate (TCP) assay which include quantification of the attached bacterial cells to each well of 96-well microtiter plates in triplicate. The amount of the attached cells can be quantified after staining with crystal violet and reconstitute of the stain in solvent and measuring the OD at 630 nm. The results showed that most of CVEC were not biofilm former and compile 20 (62.5%). The biofilm formation among CVEC compile 12 (37.5%) and among them 1 (3.125%) were strong biofilm former; 3 (9.375%) were moderate biofilm former and 8 (25%) were weak biofilm former) Figure 6.

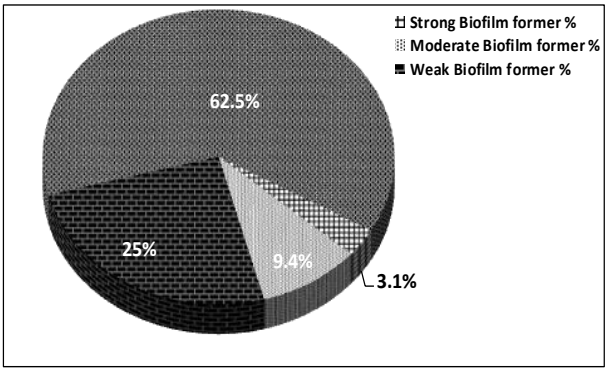

Figure 6: Biofilm formation among CVEC.

DISCUSSION

Concern phylogrouping, many studies were in accordance with our findings. Al-Saffar (2016) report that 100% of *E. coli* isolated from women with vaginitis in Hilla City, Iraq allocated within extraintestinal phylogenetic subgroup B23. Study from Al-Kut city, Iraq documented that (81.8%) of *E. coli* isolated from pregnant and non-pregnant women were assigned to group B2 (Al-mayahie, 2013). Al-Khalide *et al.* (2015), found that (58.46%) of *E. coli* isolated from high vagina and endocervix of women from Kerbala-Iraq. Obata-yasuoka *et al*. (2002) and Rashki (2014) found that 76% and 62.12% of the CVEC isolated from women with bacterial vaginosis were belong to phylogenetic group B2. It is seemed that most of the isolated *E. coli* were virulent and isolates belong to intestinal group less existence in vaginal epithelium.

Regarding antibiotics susceptibility, our results was in accordance with some findings of Rashki (2014) who found that, the resistance to cefazolin, cefotaxime and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

ceftazidime were (91.66%), (86.36%) and (45.45%) respectively. Concern resistance to cephems (cefazolin, cephalothin, cefepeim, cefotaxime, ceftazidime, cefoxitin) the following CVEC exhibited resistance to all sex members of cephems: E1, E13, E14, E15, E22-E27 and E29.

The results displayed by Qin *et al*. (2013) were approximately similar to those stated by our study. They found that, the resistance of ExPEC isolated from female were (21%, 57%, 29%, 21% and 0%) for cefazolin, cefotaxime, ceftazidime, cefepime and imipenem respectively. The huge and uncontrolled users of cephems is the main cause to emergence of resistance. Results of imipenem resistance showed that our results less than those reported by Rashki (2014) (15.5% vs 34.93%). Giray *et al.* (2012) from Turkey and Qin *et al*. (2013) from China display no resistance to imipenem and meropenem in contrast to our study and this may be due to the strict regulars and instruction for prescription of drugs in their country.

Our result have a similarity and deference at the same time with those of Qin *et al*. (2013) according to type of antimicrobial agents. The similarity is, all isolates of ExPEC were sensitive to nitrofurantion and this exactly in accordance with our finding. The difference are high resistance (differences) to aminoglycoside, (10%) to amikacin, (57%) to gentamicin, (69%) to ciprofloxacin. Soleimani *et al*. (2014) report that (21%) and (3.62%) of ExPEC isolated from patient with cystitis in Tehran were resistant to gentamicin and amikacin respectively and these results in agreement with our findings.

The most suitable explanation is the resistance to antibiotics emerged to the aminoglycosides due to focusing on them as an excellent choice for treatment of most of Gram positive and negative bacteria and no need to use carbapenems leads to late emergence of resistance to them and these facts completely in contrast to drug administration polices used in Iraq. Aminoglycosides play an important role in curing bacterial infections. Modification of aminoglycosides by aminoglycosidase enzymes is the common resistance mechanism against aminiglycosides in *E. coli* (Bellaaj *et al*., 2003; Choi *et al*., 2003).

Concern resistance to fluoroquinolones (ciprofloxacin and norfloxacin), our results in agreement with those reported by Moreno *et al*. (2006) who found that only (12%) of UPEC isolated from women with cystitis and pyelonephritis were resist to fluoroquinolones and all susceptible UPEC isolates were belong to phylogenetic group B2. Due to the increased resistance of ExPEC (especially UPEC and CVEC) isolates to trimethoprime-sulfamethoxazole, it was replace by fluoroquinolones as broad-spectrum antimicrobial agents (Gupta *et al*., 2001; Sakhuja *et al*., 2001).

The right explanation of high resistance percentage to carbapenems and low resistance percentage for fluoroquinolone among our results is the uncontrolled jumping for antimicrobial prescription. It is clear to note that the antimicrobial prescription in private sector (especially daily clinics) tend to prescribe highly effective antibiotics (like imipenem or meropenem) for short period regardless it is used as last choice treatment for complicated unresolved infections. So many physicians shift from treatment with fluoroquinolones to carbapenems incurious to emerging of resistant strains. According to the results stated above it is clear to say that the treatment with amikacin, gentamicin, ciprofloxacin, norfloxacin and nitrofurantion still possible to cure the infection caused by CVEC.

Concern biofilm formation, TCP were used due to that it regard the simple, cheapest gold standard for quantitative biofilm formation yet (Knobloch *et al*., 2002; Mathur *et al*., 2006; Hassan *et al*., 2011). The differences in percentage of *in vitro* biofilm formation among ExPEC may effected by many factors like curli formation, osmolality of medium, type of medium and expression of some bacterial protein like TolC (Hou *et al.*, 2014).

The fluctuation simple irreproducibility of all phenotypic assays may be due to the facts that: The same species may give different results upon repeated testing and the assay result depends on individual interpretation and expertise. Furthermore, small alterations in the execution of an assay may give false assay results. Consequently, identification based on phenotypic tests does not always allow an unequivocal identification.

CONCLUSION

This study conclude that, most of the CVEC belong to highly virulent phylogroup B2 and have the ability to resist multiple antibiotics and the ciprofloxacin, norfloxacin, amikacin and nitrofurantion still the best choice for treatment and CVEC have the ability to form biofilm which make the infection ruthless and hard to cure.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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