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Effect of Conjugation between *Pseudomonas aeruginosa* and *Escherichia coli* on Antibiotic Resistance

Rasha M. Al-Oqaili , Huda Z. Majeed , Basam B. Mohammed

Biology Department. College of Science, Mustansiriyah University, Baghdad, Iraq.

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Abstract: Urinary tract infection (UTI) is always connected with *Escherichia coli* bacteria; Antibiotic resistance in these bacteria is an unlimited threat in treating UTI. Plasmids are extrachromosomal elements for medically important characters, such as antibiotic resistance, in addition to be the main way of horizontal gene transfer of the in bacteria. The study involved isolating and characterizing bacterial strains using Methylene Blue (EMB) and MacConkey agar, revealing pink colonies of *E. coli* indicative of lactose fermentation. Biochemical tests showed fermentation of glucose, lactose, and sucrose, with positive results for indole and methyl red, while being negative for Voges-Proskauer and citrate utilization. *E. coli*, a common cause of urinary tract infections (UTIs), poses challenges due to unidentified genetic factors affecting pathogenicity and antibiotic resistance genes found on plasmids. Meanwhile, *Pseudomonas aeruginosa* isolates displayed beta-hemolysis and pigment production. Antimicrobial susceptibility tests indicated that *E. coli* was highly sensitive to several antibiotics, whereas *Pseudomonas aeruginosa* was resistant to ciprofloxacin but sensitive to others. The recommended treatment for uncomplicated cystitis includes nitrofurantoin and fosfomycin, with ciprofloxacin reserved for severe infections. Conjugation experiments confirmed the transfer of ciprofloxacin resistance genes, suggesting their presence on conjugative plasmids.

Keywords: *E.coli*, *Pseudomonas aeruginosa*, conjugative plasmids, Antibiotic resistance.

Corresponding author: (Email hudazuheir@uomustansiriyah.edu.iq).

Introduction

Plasmids can add new genes to cells of the host, to enhance specific characters e.g. antimicrobial resistance or host cells adhesion mechanisms (1). Plasmids are circular short extrachromosomal DNA elements which able to mobile between various host cells found in the same environment. Transformation, and bacteriophages also allow gene transfer of plasmids, but less common than conjugation do (2).

Those horizontal gene transfer (HGT) ways cleared the of plasmids contribution in adaptation ability to new ecological niches which had the basic role of pathogenic bacteria spread. The pathogens clonal radiations are always connected with the HGT-depended gaining of plasmids encoding key Antimicrobial resistance genes (e.g. *blaOXA-48*) which responsible of survival in the antibiotic-rich niches. This means that modify the host genes assortment and integrations of proteins with plasmid had its own advantage on

host adaptation to changeable environments (3).

Escherichia coli and *Klebsiella* are the two prior pathogens effecting on health of human according to the World Health Organization (WHO) reports. So, when plasmid caused antimicrobial resistance genes gaining by ExtraIntestinal pathogenic *E. coli* (ExPEC) has extended their ability of epithelial cells infection in their niches in the normal gut (4).

In this study, conjugation between *E.coli* isolated from urinary tract infections and pathogenic *P. aeruginosa* was studied, especially for antibiotic resistance and gene transfer.

Materials and methods

1. Isolation of *E. coli* bacteria

Out of 150 samples collected of UTI patients, 65 isolates were primary identified as *Escherichia coli* and were identified by its phyrnolysin on blood agar and typical colonies with iridescent "sheen" on Eosin meythylene blue (EMB) and grow on MacConky agar and form circular , convex, smooth colonies with distinct edges giving pink colour as an induction for lactose fermentation. The isolate of *E. coli* appear sensitive 100 % to the antibiotics: Nitrofurantion, Ampicillin, Cloxacillin, Amoxicillin and Ciprofloxacin.

For Ciprofloxacin stock solution it was prepared at (10) µg/ml by dissolving (0.1) gm of the antibiotic in (9) ml of distilled water ,then the amount was completed to (10) ml by distilled water according to what mentioned approval in 2003 Stephenson, then sterilized by using the Millipore filter at (0.22) µm .

2. Isolation of *Pseudomonas aeruginosa* bacteria

Sixty isolates of Gram negative bacteria were taken from Mustansiriyah University, College of Science, Biology Department. There were six isolates primarily diagnosed as *P. aeruginosa*.

The isolate of *Pseudomonas* (No. 6) produced a zone of Beta hemolysis with grape smell on blood media and pale colonies on MacConkey agar (Lactose none fermenting). Also grow on *Pseudomonas aeruginosa* agar selective medium pigments as pyocyanin and the fluorescent pigments pyoverdine were produced. The isolate *Pseudomonas* appear sensitive to antibiotic Nitrofurantion, Ampicillin, Cloxacillin and Amoxicillin but resist to antibiotic Ciprofloxacin.

3. Identification of Bacterial isolates

According to the diagnostic procedures recommended by (5), the isolation and identification of *E .coli* associated with patients under study were performed as follows

Morphological Examination

Primary examination was based on morphological characteristics (e.g. colony size, shape, color, tranparency, edge, and elevation of texture) of bacterial growth on *Pseudomonas* agar, MacConkey and Blood agar (6). The colonies were then examined by Gram stain.

Microscopic Examination

By transferring one isolated colony into a slide, fixed, after that stained with Gram stain to observe the arrangement and shape of cells (6).

Biochemical tests

The later biochemical tests were done in order the bacterial isolates identification. These tests were done according to (6) which includes.

Oxidase test

Single isolated colony was transported to a filter paper by wooden stick, and added (2-3) drops of oxidase reagent to it. The change to dark purple

color within (20-30) sec means a positive result (7).

Catalase test

The slide method was used, which done by transferring amount of purified growth by a wood stick into a microscope slide, and add drops of 3% H_2O_2 on the colonies. Forming gas bubbles refers to a positive test (8).

Indole test

Pepton water was cultured and incubated at 37°C for 24 hrs. Fifty microliter of Kovac's reagent was added and gently mixed. A positive result was referred by the presence of a red ring on the surface of liquid media.

Methyl Red Test

MR-VP broth was cultured and incubated at 37°C for 24 hrs. five drops of methyl red reagent was added and gently mixed, positive tests were bright red and negative were yellow.

Voges Proskauer Test

MR-VP broth was cultured and incubated at 37°C for 24 hrs. Then 1 ml of Baritts reagent A and 3 ml of reagent were added into the cultured broth (5 ml) and shake for 30 sec. The formation of pink to red color indicated a positive test for acetone.

Citrate utilization Test

Citrate agar slants were cultured by pure colony (age 24hr) by stabbing the bottom and streaking on the slant surface, and then incubated for 24 hr at 37°C. When color change from green to blue means a positive test. This test detects the bacteria ability to use citrate as carbon source (9).

Triple Sugar Iron test

Test was carried out by TSI agar slant inoculation with single isolated colony of bacterial growth by stabbing the bottom and streaking on the slant surface, then inoculated at 37°C for 18-24 hrs. The color change of the slant, and bottom, gas and H_2S production were recorded (10).

Urease test

Urease production was detected by inoculating the urea medium with single colony of bacterial isolates, and incubated at 37°C for (18-24) hrs. The color changes into pink which indicates the positive result (9).

Culturing on Eosin methylene blue (EMB) agar

EMB is a differential medium was used to determine the *E .coli* bacteria from other species. One isolated colony was cultured on this medium and incubated for 24 hrs at 37°C. *E. coli* bacteria grown on this medium will give a metallic green sheen (9).

These identifications were confirmed by the API 20 E test system.

1. Antimicrobial susceptibility test

All isolates were subjected for antimicrobial susceptibility according on the (11) criteria as following: About (2-4) Few colonies from 24 hr culture were transferred to 2 ml of normal saline to get the bacterial suspension and were adjusted to 0.5 McFarland turbidity (e.g. 1.5×10^8 CFU /ml). The bacterial suspension was cultured on Muller Hinton agar plates by a cotton swab and plates left to dry. Different antimicrobial discs Ciprofloxacin (10 µg), Ampicillin (10 µg), Cloxacillin (10 µg), Amoxicillin (300 µg) and Nitrofurantion (10 µg)) were put with a maximum five discs place on the surface of the medium and the plates were incubated at 37°C for 24 hours (duplicate was done for each antimicrobial). The inhibition zone diameters for each antibiotic disc was measured and interpreted by referring to CLSI values.

2. Conjugation *in vitro*

a) Inoculation of 2 tube of Brain heart infusion broth, first tube with *Pseudomonas* (Ciprofloxacin resistance) as donor cell, while the second tube with *E. coli* (Ciprofloxacin sensitive) as recipient.
b) Amount of 25 µl of recipient cell broth and put it in new tube, and add to this tube (475) µl of donor cell broth (the ratio will become (19:1) donor: recipient) , then add (4.5) ml of Brain heart Infusion broth and mix well. This mixture will be filtrated by membranous filter with pores (0.22) µm.
c) The filter paper under sterilized condition by a sterilized forceps to Brain heart Infusion agar surface (put filter paper surface that contains the bacterial growth on the upper side) and avoid air bubbles between filter paper and medium surface. Then incubate the plate in 37°C for 24 hr.
d) The filter paper under sterilized condition with growth on it to a new tube contain (2) ml of Brain heart Infusion broth and mix well to ensure bacterial growth transferring from filter paper to the broth.
e) Serial dilution from (10^1 to 10^8) were prepared, then transfer (0.1) ml form each tube and culture it by spreader on selective medium which is Muller Hinton agar containing Ciprofloxacin (30 mg /ml) and incubate plate in 37°C for 24 hr. Note that the colonies in conjugative state (donor and recipient cell through their growth on selective medium containing antibiotic).

Results and Discussion

1. Isolation and identification of *E. coli* from UTI patients

Out of 150 samples collected of UTI patients, 65 isolates were primally identified as *Escherichia coli*. The isolates identification was done depending on specific morphological, cultural and biochemical tests as mentioned by (12).

Urinary tract infections is one of the most common human bacterial infections, there are about (150–250) million cases around world each year (13). If infections of UTI studied comparing Hospital acquired infections, UTIs formed (40–50)% of bacterial infections leading to increasing morbidity then hospitalization being for a long time (14).

The existence of bacteria in the urine about ($\geq 10^5$ CFU/mL) without any clinical symptoms is referred as asymptomatic bacteriuria (ABU). Mainly UTIs are caused by *E. coli*, which in turn are the cause for (70–95) % of UTI cases (14)

The result was compatible with study of (15) who reported that *E.coli* is the main causing agent in UTIs and its one of the most commonly spread bacterial infections.

Usually a bacteria could stand behind an UTI but without connection of sex, age and location, *E.coli* is the main pathogen (16), and it is the causative agent in approximately (90)% of the cases in young-aged patients by un complicated UTI.

The identification and confirmation was done on the basis of morphological, biochemical and phenotypic characteristics. This bacterium can quickly identified as *E.coli* by its haemolysin on blood agar media and its typical colonies with iridescent "sheen" on Eosin Methylene Blue (EMB) agar and grow well on MacConkey agar forming circular, convex, smooth colonies with distinct edges besides giving pink color as induction for lactose fermentation ,this result agreed with (13).

Biochemical tests for all isolates showed fermentation of glucose, lactose and sucrose, IMViC test showed indole positive, (this test depends on the ability of the bacteria to split tryptophan to form indole which gives a red color with Kovac's reagent), positive for methyl red (a red coloration obtained following the addition of methyl red reflects the ability of the bacteria to acidify the phosphate buffered glucose peptone media to pH=4.4 or below and keep stably end products produced of fermentation of glucose in overnight culture). Negative for Voges- Proskauer (a test used to detect the ability of the bacteria to produce neutral end products (acetoin and / or diacetyl) in phosphate buffered glucose –peptone medium). Negative for citrate utilization tests (it detects the ability of the bacteria to use citrate as alone source of carbon. A positive test was obtained when the color of the medium change from green to blue with a streak of growth (17).

They have ability to react on triple sugar iron (TSI) medium. Urease Test used to detect the bacteria ability to produce urease that hydrolyses urea to ammonia and CO_2, in a positive reaction, the resulting alkalinity causes the pH indicator to turn pink (18).

Escherichia coli is one of urinary tract infection causes (19). The genetic determinants that stand behind *E. coli* pathogenicity in the urinary tract are still unidentified completely, So, management is a growing challenge(20). In addition, antibiotic resistance presence (21). Genes of antibiotic resistance are usually found on plasmids, that can replicate and contain resistance genes besides genes coded for other characters e.g. virulence and fitness (22).

Plasmids could be incompatible which makes 2 plasmids unable to keep them in the same host (23). The most important example of medically important plasmids in *E. coli* is the F plasmids, which is known by their inc loci incFI and/or incFII (24). F plasmids are spread easily, heterogenous, mobile and always stable (25). Urinary *E. coli* contain F plasmid, e.g. extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolated from urinary infection (26). In addition to the uropathogenic *E. coli* (UPEC) strain UTI89 had a hybrid F plasmid – pUTI89 – and its absence leads to a observed decrease in virulence in the mouse model during the beginning of infection (27). The pUTI89 plasmid has genes encoded to pathogenicity, besides to the conjugation system that is responsible of Mobility of plasmids (22).

Conjugation is transfer of genetic information between cells mediated by conjugative elements e.g. Pilus, enzymes (e.g. relaxosome for processing of DNA), Stability of mating pair mediated by proteins and the type IV secretion system (T4SS) (27). The *E. coli* known conjugation system is the F-type system, which depends on transfer of *tra* genes (22).

Collection and diagnosis isolates of *Pseudomonas*

The six collected isolates were primarily diagnosed as *Pseudomonas* bacteria. The bacterial isolates were cultured on different media e.g. MacConkey agar, Blood agar, and *Pseudomonas* agar in air presence followed by other diagnostic tests.

They appeared as large flat colonies that responsible of beta-haemolysis zones with odor of grape on the blood agar, besides, pale colonies on MacConkey agar (indication that lactose

non-fermenting colonies). This bacterium was capable to grow on *Pseudomonas* agar as the selective media showing pigments as pyocyanin and the fluorescent pigment pyovirdin. Microscopically Examination of *P. aeruginosa* showed Gram -ve, small rods present as a single bacteria or pairs and non-spore former bacteria.

Antimicrobial susceptibility for *P. aeruginosa*

All the isolates of *E. coli* were sensitive to ciprofloxacin (10µgl) but one isolate was sensitive 100% to Ciprofloxacin, Ampicillin (10 µg), while *Pseudomonas* were sensitive 100% to Ampicillin (10 µg), Cloxacillin (10 µg), Amoxicillin (300 µg) and Nitrofurantion (10 µg), but resistant to Ciprofloxacin (10 µg).

The results of sensitivity tests showed that *E. coli* was sensitive by 100% for the antibiotics (Ampicillin 10 µg, Cloxacillin 10µg, Amoxcillin 300µg, Nitrofurantion 10µg, and Ciprofloxacin.

UTI treatment is recommended by using: Nitro.furantoin, Fosfo.mycin Tromet.amol, and Trimetho.prim plus Sulfa.methoxazole (SXT) (28). Nitrofurantoin and Fosfo.mycin are considered as 1st line treatment for uncomplicated cystitis, But SXT is not recommended because of high bacterial resistance (29). So, Ciprofloxacin and levo.floxacin are considered as an main antibiotic for the treatment of more severe infections treatment, e.g. septicemia, and thus ciprofloxacin must be considered as an alternative, not as 1^{st} line antibiotic for the treatment of un complicated cystitis (28). Besides Ciprofloxacin was prescribed as 2^{nd} line empiric therapy in the mild and moderate pyelonephritis or complicated UTI treatment (29).

Uropathogens had been changed, even their resistance for antibiotics had observed over the former years, making them as a global concern. Resistance risk factors formation and presence of multiple-resistance pathogenic bacteria in UTIs compromise use of antibiotics, especially with broad spectrum of activity, former hospitalization; anomalies of urinary tract, use of catheter, age and recurrent UTIs (30).

Bacterial Conjugation

The study of transmission of genetic coded factors of ciprofloxacin resistance was conducted by selecting a bacterial isolate sensitive to Ciprofloxacin, while the other isolates were sensitive to Ciprofloxacin, whereas *Pseudomonas* were considered as (Recipient), (*E. coli*) which characterized with its sensitivity to Ciprofloxacin.

The results showed success of the conjugation with all isolates, after conjugation cells were cultured and colonies grown on the selective medium which contains Ciprofloxacin at concentration of 30 µg /ml. Success of the conjugation and resistance recipe transmitting indicates that this recipe may be mounted on a conjugated plasmid or on a sudden gene which ease its transmission to another cell within the same type and other species.

It was found that Ciprofloxacin resistance genes have transmitted during the conjugation test, which indicates the presence of this recipe on a conjugated plasmid which transmitted during this test.

Conclusion

The study demonstrated the successful transmission of ciprofloxacin resistance genes through conjugation, indicating their presence on a conjugative plasmid. This highlights the role of horizontal gene transfer in the

rapid spread of antimicrobial resistance, particularly among Gram-negative bacteria. Understanding these mechanisms is crucial for controlling the dissemination of multidrug-resistant pathogens and developing effective countermeasures.

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