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Treating Antibiotic Resistance Genes in *Proteus Spp*. were Isolated from Renal Stone Patients by *Crataegus rhipidophylla* and *Adiantum capillus*

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Abstract: Nine isolates of proteus spp. were isolated from 100 urine samples of renal stone patients which were the urine specimens obtained directly from Sulaimania teaching hospital laboratory, and identified according to the cultural characteristic, morphological, biochemical examination. The antibiotic susceptibility test for all isolates were conducted to nine antimicrobial agents including (Ciprofloxacin (Cip), Tetracyclin(TE), Neomycin (N), Gentamicin (CN), Erythromycin (E), Nitrofurintion (F), Naldixic acid (NA), Imipenim (IPM), Amoxicillin (AX). Plasmid analysis of these isolates showed presence are (22) Kb plasmid. Curing of antibiotic resistance genes by using methanol extracts for leave of Crataegus rhipidophylla and Adiantum capillus was performed. The minimum inhibitory concentration of these medicinal plants through methanol extracts which were 8000 µg/ml and 1200 µg/ml for Crataegus rhipidophyllaand Adiantum capillus respectively. The Sub minimum inhibition concentration (SMIC) was also determined. The results of transformation and curing experiments revealed that SMIC of Crataegus rhipidophyllaextract was cured or eliminated plasmid completely, and (SMIC) of Adiantum capillus was cured (CN, E, and AX) resistant genes.

Keywords: proteus spp., Plasmid, antibiotic resistance, transformation, curing, medicinal plants

1. INTRODUCTION

Proteus, Providencia, and Morganella; Members of the Enterobacteriaceae are agents of urinary tract and other extra intestinal infections. Proteus species are relatively common causes of uncomplicated as well as nosocomial UTI [1].

This rod shaped bacterium has the ability to produce high levels of urease. Urease hydrolyzes urea to ammonia (NH3) and thus makes the urine more alkaline. If left untreated, the increased alkalinity can lead to the formation of crystals of struvite, calcium carbonate, the bacteria can be found throughout the stones, and these bacteria lurking in the stones can reinitiate infection after antibiotic treatment [2, 3].

Antibiotic resistance" is a major problem in the world now, so the medicine manufactures yearly produce new generations of antibiotics to solve this problem, especially there are some bacteria considered a multiple antibiotic resistance as *E. coli*, *Klebsiella spp.*, *Salmonella spp.*, *Staphylococcus aureus*, *pseudomonas spp*., *proteus spp* , *providencia spp.* and others. Therefore, the recent studies towards using medicinal plants for solving this problem, by curing resistant plasmids or reducing some resistant genes which occur on plasmids DNA considered a biological, medicinal and industrial (artificial) importance in their lines [4].

The aims of this study were isolation and identification of *Proteus spp.* Collected urine from renal stone patients by cultural, morphological and biochemical tests including API 20E test, then Study the resistance of *proteus spp.* to different antimicrobials agents and then studying the effect of Extraction of *Crataegus rhipidophylla* and *Adiantum capillus* extracts on the elimination of antibiotic resistant genes in some of isolated bacteria.

2. MATERIAL and METHOD

Isolation and Identification of Bacterial strains: 100 urine specimens were directly obtained from laboratory of Sulaimania Teaching Hospital which have obtained the specimens from renal stone patients, the identification and characterization of isolates were determined on blood agar and MacConkey agar. The biochemical tests of suspected bacteria that were detected by IMVC (Indole, methyl red (MR), vogesproskauer (VP) and Simon's citrate agar), triple sugar iron agar (TSI agar) (Mast diagnostic U.K.), urease, gelatinase, oxidase and catalase test In addition to the biochemical reaction test; API 20E identification system (bioMérieux, France) was perform [5, 6].

Antibiotic susceptibility testing: Antibiotic sensitivity test was performed by applying a bacterial inoculum of approximately 1–2×10^8CFU/mL to the surface of mueller-hinton agar plate, and used disc diffusion method [7, 8]. The following antibiotic discs (drug concentration in µg) were used: Ciprofloxacin 30µg, Tetracyclin (TE)10µg, Neomycin (N) 30 µg, Gentamicin (CN)10 µg, Erythromycin (E)15 µg, Nitrofurintion (F) 100µg, Naldixic acid (NA) 30 µg, Imipenim (IPM) 10 µg, Amoxicillin (AX) 25µg that obtained from pharmacy in KOya city and used (0.5 McFarland) of tested bacteria [9].

Selection of medical plants for study: These medical plants including *Crataegus rhipidophylla, Adiantum capillus* were obtained from local market in Sulaimani city then Preparation of crude extracts performed by (Handa) [10] method.

Determination of minimum inhibitory concentration (MIC): The minimum inhibitory concentration was determined for plant extract, which inhibited bacterial growth. The following dilution were prepared for each extract (100, 200, 300, 400, 500,600,700, 800,900,1000,2000,3000, 4000, 5000, 6000) µg/ml [11]. In addition, the SMIC of medicinal plant extracts was determined and used as curing agent.

Plasmid curing with medicinal plant extracts: The plasmid curing with medicinal plant extracts was tested by streaking method; nutrient agar was used as growth medium, after sterilization and cooling at 45˚C. Final concentration of antimicrobial under study and (sub-MIC) of plant extract were added, the medium was mixed and poured in Petri dishes, and after solidification it was inoculated by streaking method with *Proteus spp*. then incubated at 37˚C for 24 hours. Next day the sensitivity and resistance of isolate *Proteus spp*. were recorded.

Plasmid DNA extraction by alkaline lyses

A single colony of bacterial isolates was grown in 10 ml of LB broth containing 50µg/ml Erythromycin and incubated at 37C˚ for 24 hr. with shaking. Bacterial cells were harvested by centrifugation at 10000 rpm for 10min, and then transferred to sterile Eppendorf tube [12].

Preparation of the competent cells

Many bacteria can be made competent by exposure to a divalent or multivalent cation, such as calcium chloride, .[13] manganese chloride

DNA uptake

100µl of prepared plasmid DNA (0.1) ml added to tube containing 0.2 ml of competent cells. The mixture was placed on ice for 30 minutes, exposed to heat shock at 42 C˚for 6 minutes [14]. After that 1 ml of fresh nutrient broth was added to transformation mixture, and incubated at 37C˚ for 60 minutes to allow the expression of the antibiotic resistant genes. All Samples of 0.1 ml from transformation mixture were spread on nutrient agar plates containing different antibiotics used, and 0.1 ml of competent cells spread on nutrient agar containing same antibiotics used as control, all plates were incubated at 37 ˚C for 24 hours as described by [15].

Electrophoresis of plasmid DNA: Agarose gel electrophoresis was performed using 0.8% horizontal slab gels in TEA buffer (pH 8.0) containing 50 mM Tris, 20 mM sodium acetate, 2 mM EDTA, and 18 mM NaCl [16] Gels were stained with ethidium bromide (2µl) and ran at 60 volts for about 1.5 h.

3. RESULTS

Identification of *Proteus spp* 100 urine specimens were directly obtained from laboratory of Sulaimania Teaching Hospital which have obtained the specimens from renal stone patients. After isolation the characteristics of isolates were studied, through culturing them on differential medium, such as MacConkey agar and blood agar. In this case, the isolates appear on the findings of non-lactose fermenting colonies with swarming on blood agar and chocolate agar plate, characteristic fishy smell, Gram negative bacilli with active motility colonies, so the lighter-colored alkaline reaction is seen on EMB agar.

The biochemical test for bacterial isolates: reduced nitrate to nitrite, positive for (urea, catalase, phenylalanine and motility) , fermented glucose (with acid and gas) and produce heavy H_2S,ferment sucrose and mannitol but do not ferment lactose, usually does not utilize citrate but hydrolyze urea and produce phenyl pyruvic acid, and negative for oxidase test [17] as shown in figure (1).

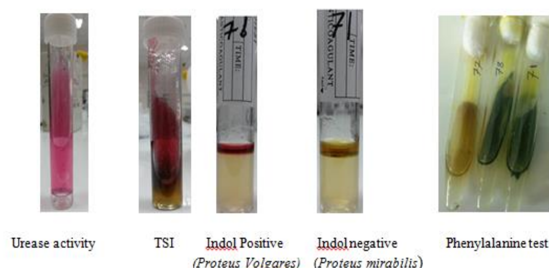

Figure (1) Proteus spp. Biochemical tests
A positive phenylalanine is determine by light to dark green color
A negative test is indicated by the absence of a green color reaction

In addition to the biochemical reaction test, API 20E identification system was performed according to analytical profile index (1999) as showed in figure (2). Thus according above study from one hundred samples seven (9%) of the isolates were identified as *Proteus spp*., and the results show two of them *Proteus Volgares* because indol test positive and others appear *Proteus mirabilis.*

Figure (1) Results of API 20E test used for identification of ***Proteus spp***

Thus according above study from one hundred samples nine (9%) of the isolates were identified as *Proteus spp*., and the results show two of them *Proteus Volgares* because indol test positive and others appear *Proteus mirabilis.* The study of [18] shows that ***E .coli*** was among the most predominant pathogenic bacteria isolated from the urine with a rate of (46.21%), while *Proteus mirabilis* were (13.25%), and other species were (40.54%).

Antibiotic susceptibility test was conducted for nine isolates, nine widely antimicrobials used (Cip, Ne, TE, CN, E, F, NA, IPM, AX) which demonstrated in table (1) and used at disc diffusion Kirby and Bauer method.

Table (1) illustrate multidrug-resistance (MDR) phenotypes that resistant to more than one antimicrobial agents and high resistance rates were observed for Erythromycin 77.77%, Tetracyclin 66.66% and Nitrfurintion 66.66%, and lower resistance rates were

observed for Ciprofloxacin 0%, Gentamycin 22.22% and Naldixic acid 22.22% as show in table (2).
The study of [19] showed that antimicrobial susceptib

Table (1) the susceptibility of *proteus spp.* isolates to antimicrobials in (mm)

Number of isolates	CIP	TE	N	CN	E	F	NA	IPM	AX
1	S	R	R	S	R	R	S	S	R
2	S	R	S	S	R	R	S	S	R
3	S	S	S	S	S	S	S	S	S
4	S	R	S	S	R	R	S	I	I
5	S	R	I	R	R	S	R	R	R
6	S	R	R	R	R	R	R	S	R
7	S	R	S	S	R	R	S	S	R
8	S	R	S	I	R	R	S	S	I
9	S	S	S	S	S	S	S	S	S

R= resistant S= sensitive I= intermediate

Ciprofloxacin 10µg, Tetracyclin(TE)10µg, Neomycin (N) 30 µg, Gentamicin (CN)10 µg, Erythromycin (E)15 µg, Nitrofurintion (F) 100µg, Naldixic acid (NA) 30 µg, Imipenim (IPM) 10 µg, Amoxicillin (AX) 25µg

Table (2) Resistance percentage rate to antimicrobial agents

Antimicrobial agents	No. of resistant isolates	Resistant %	Sensitive %
Ciprofloxacin	0	0	100
Tetracyclin	6	66.66	33.33
Neomycin	1	11.11	88.88
Gentamycin	2	22.22	77.77
Erythromycin	7	77.77	22.22
Nitroferontion	6	66.66	33.33
Naldixic acid	2	22.22	77.77
Imipenim	1	11.11	88.88
Amoxacillin	5	55.55	44.44

Plasmid profile analysis: Plasmid DNA was extracted from *Proteus spp.* isolates by alkaline lysis. Investigation was done in order to find relationship between antibiotic resistance and genetic determinants. The results revealed that five of the isolates were undergone to plasmid extraction but just one of them have one plasmid the size of this plasmid about 2200bp in the lane 3. In lane 1 showed plasmid marker 1kb (it starts from 500bp to 10000bp) for measuring the size of plasmids as showed in figure (3-A).

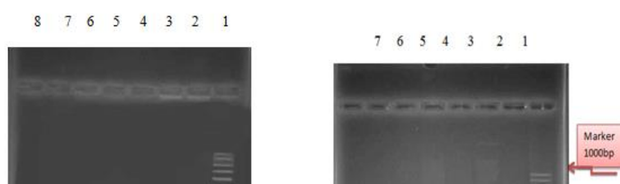

Figure (2) the plasmid profile of *Proteus spp*
Electrophoreses run at 85v for 1 hr.

[A]Lane 1: plasmid marker
Lane 3: Proteus *spp* No.2 isolate
[B] Lane 1: plasmid marker
Lane 2: Proteus *spp* No.2 isolate before transformation
Lane 3: Proteus *spp* No.2 isolate after transformation
Lane 4: Proteus *spp* No.2 after treating with Ailanthus altissima
Lane 5: Proteus *spp* No.2 after treating with Adiantum capillus
Lane 6: DH10B *E. coli* plasmid less strain

Genetic site determination of antibiotic resistance in *Proteus spp.* by genetic transformation:
Transformation process was done by adding *Proteus 2* to ***E. coli*** DH10B strain, the mixture was incubated for 24 hours. Then, it was cultured on different antibiotics at final concentration. As a result, these resistance plasmids located on Proteus 2 Amoxicillin, Erythromycin, and Tetracycline transferred to the DH10B host successfully. On the other hand, the transformation failed to grow on nutrient agar with Nitrofurantoin (F) at the final concentration. The present study showed three resistant genes transferred to competent cell such as (Amoxicillin, Erythromycin, and Tetracycline)..

Table (3): Transformation of the DNA extracts of proteus 2 to *E.coli* DH10B strain

Number of isolates	Cip	TE	N	CN	E	F	NA	IPM	AX
Pr. 2 before transformation	S	R	S	R	R	R	R	S	R
Pr. 2 After transformation	-	R	S	-	R	S	-	-	R

Characterization of plasmid DNA containing *Proteus spp.* isolates by agarose gel electrophoresis: agarose gel which was prepared by using 0.8 gm agarose powder in 100 ml TE buffer. Figure (2) demonstrated in lane 1; plasmid marker 1kb. Lane 2 Pr. 2 has one plasmid with molecular 2200bp; lane 3 Pr.2 after transformation process to recipient strain *E. coli* DH10B. Lane 4; shows Pr.2 after treating with methanol extract of leave of Hawthorn herb *Crataegus rhipidophylla* which eliminated the plasmid with its all virulence factors completely. Lane 5; shows Pr.2 after treating with methanol extract of *Adiantum capillus* and lane 6 shows DH10B ***E. coli*** plasmid less strain. Electrophorese was

run at 60V for 1.30 hr, the length of the gel is 12cm and the width is 10cm.

Curing of plasmid DNA in *Proteus spp.* isolates by leaves of *Crataegus rhipidophylla* and *Adiantum capillus* extracts : Methanol extract of *Crataegus rhipidophylla* leaves at sub MIC have good effect on eliminating the plasmid with all resistant genes and virulence factors at 8000µg/ml . While methanol extract of *Adiantum capillus* leaves at sub MIC used at 1200 µg/ml have good effect on eliminating some resistant genes that they are responsible for (E, CN and Ax) resistant genes.

Table (4): Curing effects of *Crataegus rhipidophylla* and Adiantum capillus extracts SMIC on Proteus spp. No. 2 Isolate

Antimicrobial Agents	Methanol *Crataegus rhipidophylla*	Methanolic Adiantum capillus 12mg/ml
TE	S	R
CN	S	S
E	S	S
F	S	R
NA	S	R
Ax	S	S

The bacterium is sensitive previously to the Cip, N, and IPM
R: resistance S: sensitive

4. DISCUSSION

In the present study from one hundred samples seven (9%) of the isolates were identified as *Proteus spp*., and the results show two of them *Proteus Volgares* because indol test positive and others appear *Proteus mirabilis.* This agree with the study of (Sayran, 1995) [18] that showed ***E .coli*** was among the most predominant pathogenic bacteria isolated from the urine with a rate of (46.21%), while ***Proteus mirabilis*** were (13.25%), and other species were (40.54%).

The present study showed resistant genes of Proteus 2 which located on plasmid transformed successfully to DH10Bstrain. This agree with the study of Srwa [20] showed that the transformation of two isolates of *E. coli* to *E. coli* DH5α strain was done successfully and observed in their results different kind of drug resistance genes.

The present study determined methanol extract of *Crataegus rhipidophylla* leaves at sub MIC have good effect on eliminating the plasmid with all resistant genes and virulence factors at 80mg/ml . While methanol extract of *Adiantum capillus* leaves at sub MIC used at 12 mg/ml have good effect on eliminating some resistant genes that they are responsible for (E, CN and Ax) resistant genes. The study of [21] used alcoholic extract of the dried fronds of *Adiantum capillus-veneris* such as anti-inflammatory agents successfully.

In general we found that methanolic extracts of ***Crataegus rhipidophylla leaves,*** was more active for inhibition or decreasing the antimicrobial resistance testing bacteria, comparing with extract ***Adiantum capillus.*** Also the study of [22] used three medicinal plants for curing resistant genes that located on plasmid of gram negative bacilli *Salmonella spp.* Isolates have different activity on resistant genes that located on plasmids.

The effect of tested medicinal plant extracts and acting as antimicrobial or curing effects for decreasing antibiotic resistant activity in ***Proteus spp.*** isolates may be due to its containing active components such as ***Adiantum capillus*** , the activity is may be due to their ability to form a complex with extra cellular and soluble proteins, and with bacterial walls [23] preclinical and clinical studies carried out in recent decades have shown that fruits of emblic leaf flower of hawthorn (Crataegus spp.) possess antibacterial, antidiabetic, hypolipidaemic, anticancer, anti-inflammatory, immunomodulatory, antiatherogenic, antihypercholesterolaemia, Introduction 11 gastroprotective, hepatoprotective, cardiovascular protective and neuroprotective properties [24, 25].

5. CONCLUSION

This study was concluded 9% of the urine specimens that obtained from Sulaimania Teaching Hospital laboratory which were caused by *proteus spp*. The most antimicrobial resistance agent was Erythromycin and more sensitive were Neomycin and Imipenim. So transformation process was done to the plasmid obtained from *proteus merablis* No. 2 to DH10B plasmid less strain and successfully (Amoxicillin, Erythromycin and tetracycline) resistant genes were transformed. Then resistant genes of Pr. 2 treated with two medicinal plants (*Adiantum capillus* and *Crataegus spp.*) used at sub MIC as curing agents.

REFRENCES

[1] A. H. Richard.; C. C. Pa,ela and D. F.Bruce ‘‘Lippincotts illustrated Review: Microbiology’’, 2nd Edition, 2007. (book style)

[2] F. Luzzaro, G. D. Brigante, M.M. Andrea, B. Pini , T. Giani and E. Mantengoli *et al*. **‘‘**Spread of multidrug-resistant Proteus mirabilis isolates producing an AmpC-type beta-lactamase: epidemiology and clinical management’’. *Int J Antimicrob Agents.* ;33(4):328-33., 2009. [Medline].

[3] J.S. Lewis; M. Herrera; B. Wickes; J.E. Patterson and J. H. Jorgensen, ‘‘First report of the emergence of CTX-M-type extended-spectrum beta-lactamases (ESBLs) as the predominant ESBL isolated in a U.S. health care system’’. *Antimicrob Agents Chemother*. 51(11):4015-21. 2007 [Medline].

[4] Pooja D. Gupta, Tannaz J. Birdi*’‘Development of botanicals to combat antibiotic resistance’’ Contents lists available at ScienceDirect Journal of Ayurveda and Integrative Medicine journal (8) 266-275 , 2017. (Review Article style

[5] R.M Atlas, L.C Parks and A.E. Brown Laboratory manual of experimental microbiology Mosby-year Inc.,U.S.A. 1995. (Book style).

[6] L. Warren and E .Jawetz. ‘‘Medical microbiology and immunology’’. Hall International. Six edd. Lange Medical Books/McGraw-Hill. Medical Publishing .New York. 2000, (book style).

[7] A.W.Bauer , Kirby WMM, Sherris JC, Turk M. “Antibiotic susceptibility testing by a standardized single disk method’’. *Am J Clin Pathol* ;45:493-6. 1966.Medline Web of Science Google Scholar,

[8] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A10. Wayne, PA*:* Clinical and Laboratory Standards Institute*;* 2009.

[9] National Committee for Clinical Laboratory Standards Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards: Wayne, PA. 2000.

[10] S. S.Handa, Khanuja SPS, Longo G,Rakesh DD ‘‘ Extraction Technologies for Medicinal and Aromatic Plants’’, (1stedn), no.

66. Italy: United Nations Industrial Development Organization and the International Centre for Science and High Technology., 2008. (technical report style)

[11] R. W. Bussmann,.[1] G. Malca-García,[2] A. Glenn,[1] D. Sharon,[1] G. Chait,[3] D. Díaz,[2] K. Pourmand,[4] B. Jonat,[4]S. Somogy,[5] G. Guardado,[6] C. Aguirre,[6] R. Chan,[6] K. Meyer,[1] A. Kuhlman,[1] A. Townesmith,[1] J. Effio-Carbajal,[2]F. Frías-Fernandez,[2] and M. Benito[2] , ''Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterialremedies'',J.Ethnopharmacol;132(1):101–108. 2010 (journal style) PMCID: PMC2956840 NIHMSID: NIHMS226166 PMID: 20678568

[12] H. C. Birnboim, and J. Doly. "A rapid alkaline extraction procedure for screening recombination plasmid DNA''. Nucleic Acid Res 7: 1513- 1524. (1979).

[13] T. Nancy, and T. Janine . ''Fundamental Bacterial Genetics''. by Blackwell Since Ltd a Blackwell publishing company. 2004.. 350 Main Street, Malden, MA02148- 5020 .USA

[14] W.P.M Hoekstra, H.E.N. Bergmans and E.M. Zuidweg ''Tranformation in Escherichia coli. Studies on the nature of donor DNA after uptake an interaction genetic'', Camb., 35: 281-285.; 1980.(research style)

[15] A. Puhler, and N.K. Timmis. ''Advanced in molecular genetics'' springerverlag berlin Heidelberg, New York., 1984.

[16] Pei Yun Lee,1 John Costumbrado,1 Chih-Yuan Hsu,1 and Yong Hoon Kim1 , ''Agarose Gel Electrophoresis for the Separation of DNA Fragments'', .Journal of Visualized Experiments; (62): 3923. 2012
doi: 10.3791/3923 PMCID: PMC4846332

[17] N.R. Krieg, ''Enrichment and isolation. In manual of methods for general bacteriology''. Gerhardt, P. (eds). American society for microbiology, 143-153. (1984)

[18] A.m. Sayran, ''A Bacteriological study on the incidence of urinary tract infections in rizgary teaching hospital in Erbil city''. Msc. Theses College of Science University of Salahadin. Erbil – Iraq, (1995).

[19] B. A. Adeniyi .; C.C. Amajoyi and S. I. Smith. "Plasmid profiles of multidrug resistant local uropathogenic *Escherichia coli*, *Klebsiella spp., Proteus spp.* And *Pseudomonas spp.* Isolates" Journal of Biological Sciences 6(3) 527-531, (2006). (journal style) ISSN 1727-3048

[20] A. M. Srwa and K.K. Adel, ''Site Determination of Antibiotic Resistant Genes in *E .coil* Isolated from Different Sources''. Kirkuk Scientific studies journal. Volume 4 Number 3 ; (2009). ISSN 1992-0849

[21] Z. Zedan Ibraheim, S. Amany Ahmed, and G. Yaser Gouda, ''Phytochemical and biological studies of Adiantum capillus-''. veneris L. Saudi Pharm J., 19(2): 65–74 ;2011. doi: 10.1016/j.jsps.2011.01.007

[22] Muhammad O. Muhammad, Srwa A. Muhammad and Ibrahim S. Hamad, ''Bacteriological and Molecular Genetic Study of Salmonella typhi Isolated from Patient with Enteric Fever'', JOURNAL OF KOYA UNIVERSITY, 2009. ISSN 2073- 0713 No: 12

[23] A. Smith-Palmer, J. Stewart and L. Ftfe. ''Antimicrobial propertiesof plant essential oils and essences against five importantfood- borne pathogens in food Microbiology'', 26: 118-112, 1998.

[24] K.H. Khan,. ''Role of Emblica officinalis in medicine'', a review Botany Research International, 2, 218–228, 2009.

[25] M. Krishnaveni, and S. Mirunalini, ''Therapeutic potential of Phyllanthus emblica (amla) the ayurvedic wonder''. Journal of basic and clinical physiology and pharmacology, 21, 93–105, 2010.