GENETIC EVALUATION OF PHENAZINE SYNTHSIZED BY *PSEUDOMONAS AERUGINOSA* ISOLATED GENITAL TRACT OF FARM ANIMALS

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ABSTRACT

The present study was carried out to isolate of *Pseudomonas aeruginosa* from the genital tract of some farm animals in Baghdad city to study the genetic and the ability of these isolates to produce phenazine pigment. For this purpose 240 vaginal and uterine samples were taken from 20 cows, 20 ewes and 20 goats postpartum. The result refer to that 203 samples gave positive bacterial growth. Distributed as follows 54 cows, 73 ewes and 76 goats. Depending on cultural, microscopic and biochemical characteristics, 17 isolates of *P. aeruginosa* were obtained. Addition to using differential and selective media to study the ability of these isolates to produce pyocyanin pigment. The results showed that all of *P. aeruginosa* were produced pyocyanin pigment in various amounts. Identification of phenazine was done using thin layer chromatography and high performance liquid chromatography. Results indicated that the compound separated from liquid culture was characterized as a phenazine. Genetic analysis for *P. aeruginosa* isolates was conducted to determine the location of genetic determinants for production of phenazine. Results showed that 8 isolates of *P.aeruginosa* had one plasmid band and 3 isolates had two, while 6 isolates had no bands which cured by using 275 µg /ml of ethidium bromide. The cured cells of isolates were examined for their ability of phenazine production. the cured cells continued to produce phenazine pigment. Amplification of chromosomal *phz* genes of isolates were done using PCR with specific primers. Bands of 1110 bp were characterized as related to the *phz* genes.

Key words: Phenazine, *P.aeruginosa,* genital tract, PCR, farm animals.

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التقييم الوراثي للفينازين المنتج من بكتريا *PSEUDOMONAS AERUGINOSA* المعزولة من القناة التناسلية في حيوانات المزرعة

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المستخلص

اجريت الدراسة لعزل *P. aeruginosa* من القناة التناسلية لبعض حيوانات المزرعة في مدينه بغداد لدراسة وراثة وقدرة هذه العزلات على انتاج صبغه الفينازين. لهذا الغرض تم اخذ 240 عينه من المهبل والرحم من 20 بقرة و 20 نعجة و20 ماعز بعد الولادة. اشارت النتائج الى ان 203 عينه اعطت نمو بكتيري ايجابي، حيث وزعت العينات على النحو التالي 5 بقرة و73 نعجة و76 ماعز. واعتمادا على الخصائص الزرعية والمجهرية والبايوكيميائية تم الحصول على 17 عزلة من *P. aeruginosa* اضافة الى استخدام اوساط زرعية انتقائية لدراسة قدرة هذه العزلات لانتاج الصباغ بايوسيانين. واظهرت النتائج ان كل عزلات *P. aeruginosa* انتجت صبغة البايوسيانين بكميات مختلفة وتم تشخيص الفينازين المنتج باستخدام تقنيات TLC وHPLC حيث اشارت النتائج الى ان المركب الذي تم فصله من المزرعة السائلة هو صبغة الفينازين. اجري التحليل الوراثي لعزلات *P. aeruginosa* لتحديد موقع المحددات الوراثية لانتاج الفينازين. واظهرت النتائج ان 8 عزلات من *P. aeruginosa* كان لديها بلازميد واحد و3 عزلات كان لديها بلازميدين، في حين ان 6 عزلات لم يكن لها بلازميد والتي حيدت باستخدام 275 ميكروغرام /مل من ايثيديوم بروميد. تم فحص قدرة العزلات المحيدة على انتاج الفينازين واتضح ان العزلات المحيدة واصلت في انتاجها لصبغه الفينازين مما يدل على ان الجينات المسؤولة عن انتاج الفينازين محموله على الكروموسوم، كما تم تضخيم جينات *phz* الكروموسومي باستخدام تقنية PCR. اوضحت النتائج ان العزلات انتجت حزمة طولها 1110 bpوالتي تمثل الموقع الكامل المسؤول عن تصنيع الفينازين (*phz genes*)

كلمات مفتاحية: الفينازين، القناة التناسلية، استخلاص الدنا، تفاعل سلسلة البوليمرات، حيوانات المزرعة.

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INTRODUCTION

The flora of genital tract are made of many of bacterial species that varies throughout the life cycle of the farm animals (12). The infections of female genital tract in farm animals are often due to opportunistic secondary invaders (25). Pathogenic microorganisms could gain access into the female genital tract, due to inappropriate manipulation at parturition or treatment of retained fetal membranes resulting in infertility (30). A variety of bacteria have been isolated from female genitalia in farm animals which among it *P.aeruginosa* (8). *P.aeruginosa* is a bacterium that capable to cause deferent diseases in farm animals. The presence of such type of microorganisms may led to failure to the reproduction activity of the animal because of the infection (14). There are many researchers reported that many types of bacteria inhabited the efficiency of the genital system of animals (18) which may by pathogenic and cause disease, one of them is *P.aeruginosa*. *P. aeruginosa* was a common environmental Gram-negative bacillus it was an opportunistic pathogen as well, was known for its ability to produce pigments, which were toxic to numerous bacteria, fungi and damages mammalian cells (16). In animals, the versatility enables the organism to infect damaged tissues and it caused many diseases, in cattle such as mastitis, uterine infections, enteritis, arthritis, respiratory infections and botryomycosis(20). Also many researchers isolated *P. aeruginosa* from females genitalia of goats, ewes, cows and camels (2,1,6). Phenazine was recognized as a groups of the most significant metabolic products which created by *P. aeruginosa* (23). More than 100 various phenazines structural derivatives were recognized in nature, and more than 6000 compounds which owns phenazine as a central moiety have been manufactured (17). These derivative metabolites were created by a diversity of bacteria, predominately Pseudomonads and were calculated intensively virulence (31). Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi (21). Genetic study of phenazine was done to determine biosynthetic genes for production of phenazine derivatives. (22) find that the synthesis of phenazine-1-carboxylic acid was controled by seven gene locus on chromosomal DNA of *P. aeruginosa*. Most studies on phenazines have focused on chemical modifications at one or more positions of the aromatic ring, and most are due to one or a few terminal modifying enzymes. These differences in phenazine structure extend their biological functions and therefore these modifications may determine the ecological niche in which the bacterium occupies (27). The present study aimed to perform genetic evaluation of phenazine produced by *P. aeruginosa*

MATERIALS AND METHODS

Samples

A total of 240 vaginal and uterine samples were collected from (20 cows, 20 ewes and 20 goats) during the postpartum period were examined to determine the normal bacterial flora of the uterus and vagina (3). Transcervical swabs were collected from the uterine body of each animal by using validated method (26).

Inoculation

The uterus and vaginal swabs collected were inoculated the cultures media and the material was streaked with a bacteriological loop for 5 dilutions of the inoculums. These plates were incubated in aerobically at 37C˚ for 24 hours and after incubation period the findings were recorded.

Identification of *P. aeruginosa* isolates

The *P. aeruginosa* isolates identified according to colony morphological and biochemical characters and gram stain. The biochemical screening include: IMVC, oxidase, catalase, coagulase test, TSI, urease production, gelatein liquefaction, hemolysis test on sheep blood agar and different carbohydrates utilization, in addition to culturing on selective media included; cetrimide agar and pyocyanocyl agar. The isolation and identification of bacteria was done by using analysis to the methods of (32).

Production and Identification of phenazine product

Cultivation of *P. aeruginosa* isolates were done by using modification of Pseudomonas P medium (PSP) as indicated by Frank and DeMoss (10). Phenazin product has been detected by used of TLC technique which described by Genevieve *et al*., (11) with some

modifications. The sheet (silica gel 60f-254, 0.2 mm, layer thickness and aluminum support, size 20×20 cm, Spain). The plate was taken away and leave to dry and checked by using UV. light at 254 nm, as well as R_f values were calculated. The high performance liquid chromatography (HPLC) technique (Shimadzu, Japan) with mobile phase 100% acetonenitrile was used to detection of phenazine. The flow rate was 0.5 $ml.min^{-1}$. The temperature of column was preserved at 30 °C. the peaks gained have been compared with the standard phenazine (Sigma U.S.A).

Extraction of chromosomal and plasmid DNA

DNA Extraction was done according to manufacturer's instructions (Fermentus company, Malaysia).

Curing of plasmid DNA

Serial concentrations of ethidium bromide (100, 125, 150, 175, 200, 225, 250, 275, 300 µg/ml) were added separately to sterilized test tubes containing 10ml of nutrient broth which inoculated with 0.1ml of freshly prepared culture of *P. aeruginosa* isolates. Growth density of bacterial isolates was observed visually and compared with the control. Colonies were examined for plasmid curing by extraction of plasmid DNA and examined by electrophoresis to investigate loosing of plasmids from the original isolates, then the curing colonies were examined for production of pigment by culturing on PsP medium.

Chromosomal DNA manipulation by PCR technique

A chromosomal DNA of *P. aeruginosa* isolates containing the entire *phz* locus responsible for phenazine production, was amplified by PCR by using phz-forward primers (5'TAAGGATCCGGTAGTTCCAAGCCCCAGAAAC3') and phz-reverse (5'CACATTTGATCTAGATGGGTCACGGCTATTCAG3') (BioCorp company, Canada). The amplification was carried out using 20 µl reaction mixture containing from 10X reaction buffer 2 µl, HF DNA polymerase 1 U/ 20 µl, dNTP mix 300 µM, Primer F 0.5µl, Primer R 0.5µl, MgCl2 (25 mM) 1.6µl, Dimethyl sufoxide 3% used as PCR enhancer 0.6µl, Genomic DNA 5 µl. Amplification was performed in a programmed thermal cycler were initial denaturation 94C^o for 3min followed by 35 cycle of 94 Co for 30 second, 62 C^o for 1min, 72 C^o for 1min, final extension 72 C^o for 10 min, then amplified DNA fragments were examined by utilizing electrophoresis in agarose gel (1.5%). Gels have been stained with ethidium bromide and were photographed by using gel documentation system with UV light.

RESULTS AND DISCUSSION

Identification of *P. aeruginosa*

A total of 240 samples collected from vaginal and uterus samples were taken from 20 cows, 20 ewes and 20 goats. The result refer to that 203 samples gave positive bacterial growth. Distributed as follows 54 cows, 73 ewes and 76 goats. Isolates were able to grow on cetrimide agar and suspected to be belonging to the *P.aeruginosa*. (Table 1)

Table 1. Numbers of vaginal and uterus swabs of farm animals which gave positive results

Animals	Total No. of vaginal and uterin samples	Positive samples	No. of P.aeruginosa isolates
Cows	80	54	1
Ewes	80	73	9
Goats	80	76	7
Total	240	203	17

Production and Identification of phenazine product

P. aeruginosa isolates were identified previously, to evaluate their ability for produce pyocyanin by growing on Pseudomonas P medium. Prior separation of phenazine from the cultural medium of isolates were done by TLC with the solvent system chloroform: methanol (9:1 v/v). The pigment was visualized under UV. light at 254 nm. Results showed that the phenazine was produced by isolates giving a band with R_f 0.71 as compared with the standard phenazine, these bands were related to phenazine. Also we used a typical separation of phenazine by HPLC. A standard phenazine represented by a peak with retention time of 7.9 min. HPLC test of the phenazine from *P. aeruginosa* isolates disclosed the existence of idealistic peak maxima at 375 nm. Three peaks were detected

with retention times of 7.82, 7.95 and 8.3 minuts compared with standard phenazine.

Genetic evaluation of phenazine

The localization of genes, whether chromosomal or plasmid born, was studied for ====determination of genetic determinants responsible for phenazine synthesis in isolates. For achieve this goal, isolation of plasmid DNA was done as first step. Plasmid profiles of the results in figure (1) showed there were 8 isolates of *P.aeruginosa* had one plasmid band (1, 2, 3, 4, 5, 6, 7 and 8) and 3 isolates had two (10, 11 and 12) while 6 isolates had no bands (9, 13, 14, 15, 16 and 17). To specify whether this plasmid responsible for phenazine production, a experiment was done. *P. aeruginosa* isolates were grown in nutrient broth containing gradual concentrations of ethidium bromide to determine the sub-lethal concentration. Results showed that the sub-lethal concentration of ethidium bromide was 275 µg/ml. The isolates lost their plasmid after curing in that concentration. Phenazine production for isolates were examined, in order to make evidence that this plasmid was not responsible for phenazine production. The results indicated that these isolates continue to produce phenazine over with the loses of their plasmid which indicate neither structural genes nor regulatory genes of phenazine production localized on the plasmid.

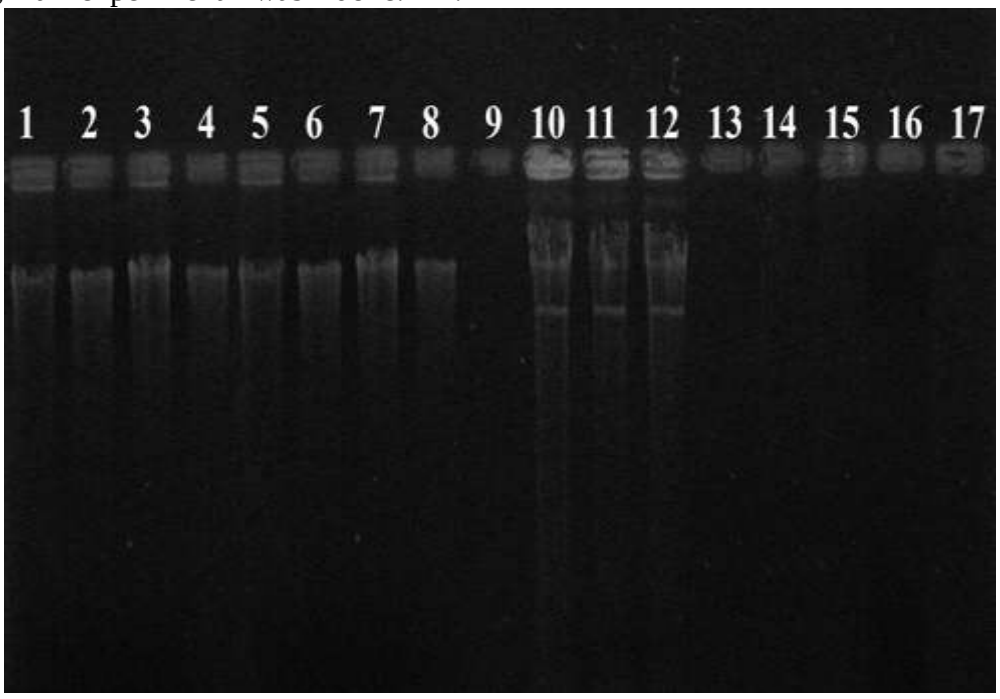

Figure 1. Plasmid DNA profile of *P. aeruginosa* isolates migrated on agarose gel (1%) in TBE buffer at 5 v/cm at 1hr

Amplification of phenazine synthesis genes

An attempt was made to localize the genes responsible for phenazine synthesis. Accordingly these loci genes (phz ABCDEFG) were amplified using specific primers (PCA1 and PCA2) along with PCR cycler and optimized specific program. The specificity of phenazine primers was reported in earlier studies by Mavrodi *etal*., (15) and Anvari, 2010 (23). The products were then electrophoresed on 1% agarose gel stained with ethidium bromide and visualized under UV. light. Results of the experiment were shown in figure (2) which indicated that amplified DNA from *P. aeruginosa* isolates showed the presence of one band of 1110 bp as compared with marker DNA (ladder DNA).

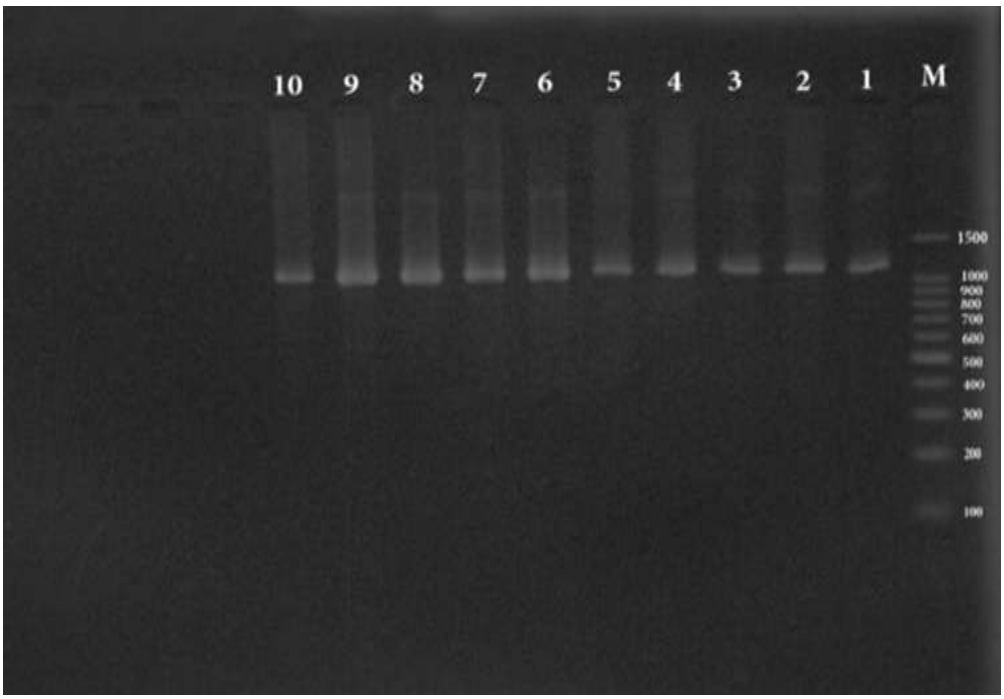

Figure 2. Agarose gel electrophoresis of the PCR products amplified. Lane M: DNA 100-bp ladder marker. Lane 1-10: DNA of *P.aeruginosa* isolates amplified by PCA1 and PCA2 primers (phz- forward and phz reverse), electrophoresis carried on 1.5% agarose gel, 5V/cm at 1.5 hr

Identification of *P. aeruginosa*

All isolates grown on cetrimide agar were subjected to the cultural, microscopical and biochemical characterization contrasted with schematic scheme suggested by Holt *etal*., (15). *P.aeruginosa* produced variety of pigments of which phenazines comprise the most important one, that pigments a water soluble blue green phenazine compound which have different biological activity related to similarity in the chemical structure to flavoproteins, isoalloxaize, flavin mononuleotide and flavin adenine dinucleotide compounds which used to phytopathogens (33).

Production and Identification of phenazine product

The results showed that the phenazine was produced by isolates giving a band with R_f 0.71 as compared with the standard phenazine. These bands were related to phenazine. The obtained results were similar to those detected by Saosoong *etal*., (29) who use TLC assessment to purify phenazine with R_f 0.70. The HPLC it is an important method for effective separation and characterization of metabolites of different organisms as long as standard was available and suitable column. That technique used by many scientists for detection and characterization of phenazine and its derivatives; therefore, Hernandez *etal*., (13) used HPLC with a C18 column to develop a rapid method for detection of phenazines, , and Anjaiah *etal*., (4) used HPLC for detection of phenazine as antifungal substance. So we used that method for detection of phenazine

Genetic evaluation of phenazine

Plasmids are important in carrying a number of genes encode traits for antibiotic and heavy metal resistance, also may generated virulence factors that allow a bacterium to colonize a host and defeat its defenses, and may has particular metabolic functions that give the bacterium to use a particular nutrient, including the capability to degrade toxic or recalcitrant organic compounds (19). The researcher Raja and Selvam, (28)found that *P. aeruginosa* contain one plasmid band, that is what we found in some isolated bacterial, while other isolates have two band of plasmid these results agreed with Dexi *etal*., (7). In order to specify the relationship between the plasmid DNA of *P. aeruginosa* of isolates and their relation with production of phenazine. Attempts were made to cure such plasmid, curing was done using ethidium bromide as an intercalating, the mode of action of agent in curing the plasmid DNA based on its ability to inhibit DNA replication during cell division without effect on chromosomal DNA

replication. All of curing isolates continue to produce phenazine, which indicate the structural genes and regulatory genes of phenazine production localized on the chromosome. Many researchers have shown that the genes responsible for biosynthesis of phenazine carried on chromosomal DNA, for instance Mavrodetal., ;Fitzpatrick,(15,2) according to Pseudomonas contain a conserved seven-gene phenazine operon (phzABCDEFG) carried on the chromosome which required for conversion of chorimsic acid to the phenazine. On the other hand, Mavrodi *etal*., (24) suggested, there was in *P. aeruginosa* a complex pyocyanin biosynthetic pathway contains of two loci in charge of produce of phenazine in addition to three genes encoding for unique enzymes participatory in the transformation of phenazine to the other derivatives

Amplification of phenazine synthesis genes

The results shown in Figure 2 indicated that amplified DNA from *P. aeruginosa* isolates presence one band of 1110 bp as compared with marker DNA. The results of the present study agreed with Anvari, (5) and Mavrodi *etal*., 2001 (22) who found that all of *Pseudomonas* isolates produced a band of 1110 bp, as compared with the band amplified from standard strain of *P. fluorescens* 2-79, and hybridized with 6.4 kb DNA probe including the whole *phz* locus when this band eluted and cloned in *E. coli* DH 5α, the positive was found productive, therefore; he suggest the fragment (1110 bp) it's the entire locus of phenazine biosynthesis

It can be conclude the possibility to determination and separation of phenazine from culture of *P. aeruginosa* by thin layer and high performance liquid chromatography. In addition to Genes for phenazine production (phz) which were localized chromosomally and the fragment (1110 bp) is entire locus of phenazine biosynthesis when amplified by PCR help to enhance the reproductive efficiency of farm animals

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