Multidisciplinary

Identification molecular biology for proteus mirabilis

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Abstract
After 4 hours of incubation, when the homoserine lactone concentration was at its greatest, was the best time for homoserine lactone accumulation. Gene-specific primers were used to genotype the Proteus isolates (16SrRNA and rpoB). The 16SrRNA sequencing of Proteus isolates indicated between 3% and 8% nucleotide alterations, and the phylogenetic tree created suggested that Proteus penneri and Proteus mirabilis are related, with both species sharing a phylogenetic branch.

Keywords: 16SrRNA**,** rpoB**,** proteus**,** molecular biology

Introduction

Adaptation of microorganisms to antibiotics has caused the spread and then persistence of drug resistance, which is currently a major public health problem, so it was urgent to discover new drugs with antimicrobial activity. In recent years, there has been increasing notice of organically active compounds comprising antioxidants from plants and other natural sources (Cai, H. Y.,. 2003.)Molecular genetics analyzes hereditary molecular variations, mainly in DNA sequences, to obtain information about the evolutionary relationships of an organism. 16S rRNA gene sequencing has been widely used as a molecular clock to estimate relationships between bacterial phylogeny (O'hara *et al*. 2000, Ronald 2003), but recently it has also become important as a method for identifying bacteria unknown up to the genus or species level. Advances have been made in automating and reducing detection times using biochemical methods, however, biochemical identification is not accurate for determining the genetic variations of microorganisms. The 16S rRNA has properties that predetermine it as global marker of evolution. There were regions on 16S rRNA that were completely conserved and then others, which were variable. Comparing the differences in the primary sequence of this 16S rRNA gene has been an excellent input for studying evolutionary changes and then the evolutionary linkage of organisms (Ronald 2003, Kim *et al.* 2003).. The rpoB gene emerged as a candidate core gene for phylogenetic analyzes and then identification of bacteria, especially when studying closely related to isolates. RpoB sequencing enables an efficient estimation of bacteria G+C% content, DNA-DNA hybridization value and then average nucleotide identity (percentage of total genetic sequence shared between two strains) when taxonomic relationships are constant. Together with the 16S rRNA gene, rpoB helped identify new bacterial species and then refine bacterial community analysis (Krishnan, T., Yin, W. F. and Chan, K.G. 2012). The Aim of Study to characterize the clinical isolates of *P. mirabilis* in terms of the molecular and phenotypic, isolation and identification of *P. mirabilis* by biochemical tests. Detection of some virulence factors genes and detection of adhesive factors genes using genetic markers.

Sample collection

Samples were collected by appropriate methods to avoid any possible contamination (Collee *et al*. 1996). Urine samples were generally collected of patients with UTI (catheter and non-catheter patients). Mid-stream urine samples were collected in sterile containers with screw caps, then urine samples were inoculated onto culture medium and incubated aerobically at 37 °C for 24 h.To collect sample, swab the wound by gently rotaing a sterile calicum alginate or rayon swab between your fingers.

Antibiotic sensitivity test

The antibiotic sensitivity test for isolates was determined, using the Power Kirby disc diffusion method on Muller-Hinton agar. The plates are incubated overnight at 37 °C, the area of bacterial growth inhibition is used as a measure of susceptibility, being large zones of inhibition indicate that the organism is susceptible to infection, while areas of inhibition indicate little or no resistance. An interpretation of the median is given for the regions between the acceptable cutoffs for the other interpretations.

Identification of bacteria by phytic system

Microorganisms are also identified by biochemical methods in the Vitek system (Pincus 1998).

Comment preparation

A sterile swab or applicator stick is used to transfer a sufficient number of colonies from a pure culture and suspend the microorganisms in 3.0 mL sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm glass test tube (polystyrene). The turbidity of the suspension is adjusted according to the turbidity of a gram-negative McFarland (0.50-

0.63) and is measured with a Densichek turbidity meter.

Vaccination

Pure colonies are inoculated in saline in labels which are controlled by turbidity measurement. These cards contain different biochemicals broth in reaction cells and one negative control cell to assess growth and viability of the suspension. The labels are inoculated with suspensions of microorganisms using an integrated vacuum device. A test tube containing a suspension of microorganisms is placed in a special rack (cassette) and an identification tag is placed in the adjacent slot while the transfer tube is inserted into the corresponding suspension tube. The filled cassette is manually placed in the vacuum chamber station. After vacuum is applied and air is reintroduced to the station, the suspension of the organism is pushed through the transfer tube into the microchannels. Conventional catalase, coagulase and oxidase tests (when applicable) and gram stain results are required prior to vaccination of cards.

Seal and custody card

The vaccinated cards are passed by a mechanism that cuts off the transfer tube and seals the card before being loaded into the carousel incubator. All types of cards are incubated online at 35.5 + 1.0 °C. Each card is taken out of the carousel incubator once every 15 min, transmitted to the optical system for reaction readings, and then returned to the incubator until the next reading time. Data are collected every 15 min during the entire incubation period. Incubation times vary from 2 to 15 hdepending on the growth rate of the organism. The Vitek-programmed computer determines whether each well is positive or negative by measuring light attenuation with a scanner. When the incubation period is complete, the reactions are automatically analyzed and the identification is printed.

Mannose-Resistant Hemagglutinin (MRHA) assay

Hemagglutination was detected by clumping of red blood cells by bacterial inactivation in the presence of D-mannose. This assay was performed according to the direct bacterial hemagglutination test, slide method and mannose-resistant hemagglutination tests (Old and Adegbola 1985). Only *P. mirabilis* strains were inoculated in 1% nutrient broth and incubated at 37 °C for 48 h for complete quenching. A panel of red blood cells was selected by obtaining blood from a human (blood type "O"). Red blood cells that were then washed three times in normal saline and 3% suspension composition in fresh saline. They were used immediately or within a week when stored at 3-5 °C. One drop of suspended was added to a drop of culture broth and the slide was shaken back and forth at room temperature for 5 min. Mannose-resistant hemagglutination was detected by the presence of 3% of group 'O' of human RBCs haemagglutination in the presence of 2% mannose (Vagarali *et al.* 2008). Bacterial agglutination of red blood cells treated with tannic acid (MR/K agglutination) was performed as previously described by (Ong *et al.* 2010) to detect the expression of type 3 fimbriae.

Genotyping assays

Extraction of DNA from gram-negative bacteria

This method was performed according to the genomic DNA purification kit supplemented by the manufacturer (Promega, USA).

Genomic prep mini spin kit protocol for elustra bacteria purification from the genomic DNA of gram-negative bacteria

Harvest the bacterial culture

Transfer 1 mL of overnight bacterial culture (600 ≤ 4.0) to 1.5 mL micro centrifuge tube.And Spun for 30 sec at full speed (16,000 × g) in a microcentrifuge. As much supernatant as possible is removed by aspiration without disturbing the cell pellet.

RNA removal

5 µL of RNaseA (20 mg/mL) was added to the sample and mixed by vortexing for 10 sec. Rotate 5 sec at 1000 x g to collect the sample at the bottom wind instrument; Incubated for 15 min at room temperature.

Detection about some factors ferocity and genes resistance for antigens vitality by PCR

Ignition

Designed factors ferocity *P. mirabilis* and the prefixes genes resistance antibiotics vitality at this is studying using design NCBI GenBank and the MP Primer Across Internet. Gene regions used in this study were given in Table 3.4.

Table 1: Gene regions used in this study

Goal genes	Sequence(5′-3′)		Producer size
ureABC gene	F	5'-TGCGGGAATGGCGGTTCGTT-3'	489 bp
	s	5'-GCCAGCAACGACTTCAGTGCCA-3'	
Florida gene	F	5'-AGGTTGTATCTGGGTGCCGA-3'	247 bp
	s	5'-GCTGACACCGCGCTGCATGCTGTCT-3'	
Zappa gene	F	TTTCCCATGCAATCAAGGC 5' 3'	308 bp
	s	5'-CTTGGAAGTGGGCGCCTGTT-3'	

* F: Forward Primer, R: Reverse Primer

Virulence genes of bacterial ısolates

Haemolysin production

Iron gain is essential for survival of invasive strains *P. mirabilis*. Producing an iron acquisition system, the hemolysin gene was tested in *P. mirabilis* isolated from urine samples. The Hly gene region was amplified by PCR using Hly primer

pairs. That associated with pathogenicity islands was present in all isolates (100%) and the amplicon band size was 120 bp, and the results are shown in Figure 3.1.

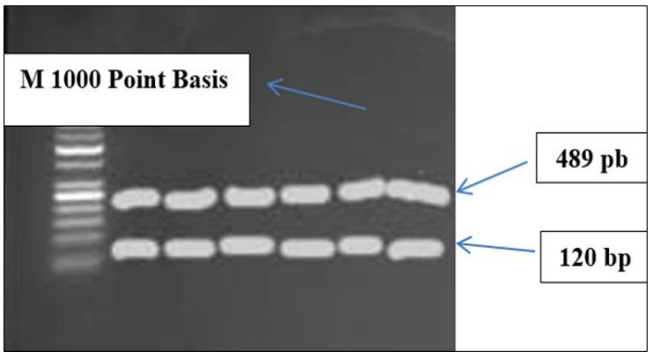

Fig 1: Gel electrophoresis of the polycon amplicon product. M: marker; No. of the *P. mirabilis* isolates, hly gene amplicon size: 120 bp and ureABC gene amplicon size: 489 bp

Examination of these positive isolates with the hly gene also for confirmation ability on extracellular hemolysin production on blood agar and it was found that all isolates (100%) had the ability to produce extracellular hemolysin. The results are in agreement with Bahraini *et al*. (1991), Sosa *et al.* (2006), and Friday *et al*. (2011) who showed that all isolated (100%) *Proteobacteria* from different clinical sources showed hemolysis on blood agar plates, but Mishara *et al*. (2001) found that 85.14% of *Proteus* isolates produce beta hemolysis while other isolates produce alpha hemolysis on agar plate. Other results reported by Mansouri and Pahlavanzadeh (2009) indicated that all *P. vulgaris* strains had hemolytic activity but at a much lower level than *P. mirabilis* strains. Thus, *P. vulgaris* strains were significantly less efficient at invading cells than *P. mirabilis* strains.

Hemolysin is a virulence-related factor for many bacterial species including *Proteus* (Mansouri and Pahlavanzadeh 2009). The function of hemolysin is to form pores in target host cells (Chalmeau *et al.* 2011). It has been shown that hemolytic activity helps *Proteus* to spread into the kidneys during infection (Coker *et al.* 2000). This is probably mediated by the increased ability of hemolytic *Proteus* cells to invade host tissues (Morgenstein 2006).

Production of extracellular proteases

These isolates have the ability to produce extracellularly enzyme protease after 24 h of incubation. A clear halo was detected from a transparent area around the colony after adding 3 mL of 5% trichloroacetic acid. The presence of the zapA gene was tested in these isolates, and we obtained positive results in 90% selected isolates and the amplicon size of the amplified gene was 308 bp, the results are shown in Figure 2.

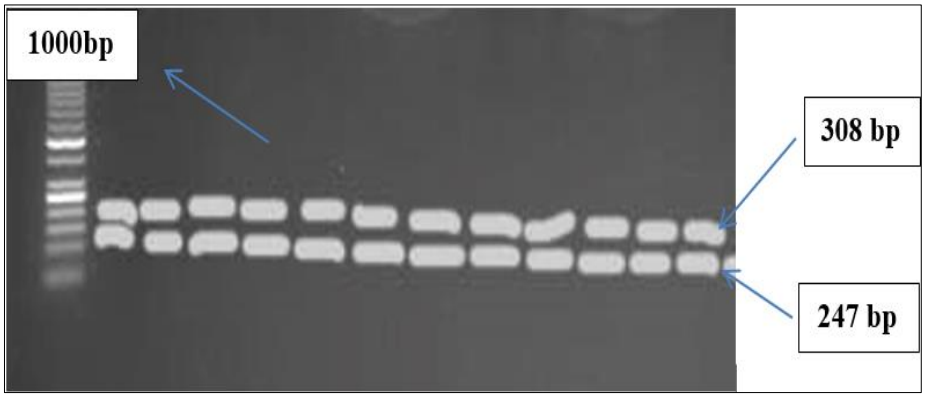

Fig 2: PCR product gel electrophoresis of zapA product and fla amplicon. M: marker; No. of the *P. mirabilis* isolates, the zapA gene amplicon size: 308bp and the fla gene amplicon size: 247 bp.

As shown in Figure. 2 only one isolate did not give a positive result about for ZapA even though the same isolate gave a positive extracellular result produce to protease. This means that there are other genes involved in the production of the protease.

The results of our study are congruent with those obtained by Coker *et al.* (2000) and Friday *et al*. (2011) who indicated that (100%) of the protus species produced the protease, but it did not agree with the results obtained by Al-Baghdadi *et al*. (2009) who stated that *Proteus* isolates were not show protease activities. The C-terminal end of zapA has a motif indicating that it is likely to be exported by a member of the large ABC family of transporters (Ruiz *et al*. 2005). Alkaline pH is optimal for the activity of many of these types of proteases, which are often present due to the urease activity of *P. mirabilis*. Unlike most IgA proteases, which only IgA breaks down in the hinge region, zapA completely degrades IgA (Nielubowicz 2010). ZapA has also been shown to be able to cleave many other proteins found in the urinary tract, including: complement components, cytoskeleton elements, and antimicrobial peptides (Aneas *et al*. 2001).

Similar to other toxins produced by *Proteus*, zapA production is increased in swarm cells (Poore and Mobley 2003, Alamuri and Mobley 2008). During infection, zapA is produced and activated, which degrades IgA in vivo. ZapA has also been shown to be essential for infection in an ascending UTI model (Nielubowicz 2010).

Genes adhesive agents

To detect the Mrp, MrkA, Uca and Atf fimbrial genes, PCR was performed using tailored specific primers and genomic DNA from each *P. mirabilis* isolate. PCR amplification of

yeast genes indicated that all isolates carried those pentameric genes. In all cases, *P. mirabilis* Mrp, MrkA, Uca and Atf fimbrial genes that were amplified by PCR showed the expected sizes (565, 416, 580 and 382 bp, respectively) and these results are shown in Figures 3, 4, .5 and .6 respectively. Hemagglutination assay results showed that all isolates (100%) adhere to human red blood cells in the presence of mannose and that means all *P. mirabilis* isolates have Mrp and Mrk genes. These results were higher than to reported by Mishara *et al.* (2001) who found 91 of 148 (61.49%) volatile isolates gave a positive result for CFA in the presence of D-mannose. Also, they were in agreement with the findings of Sosa *et al.* (2006).

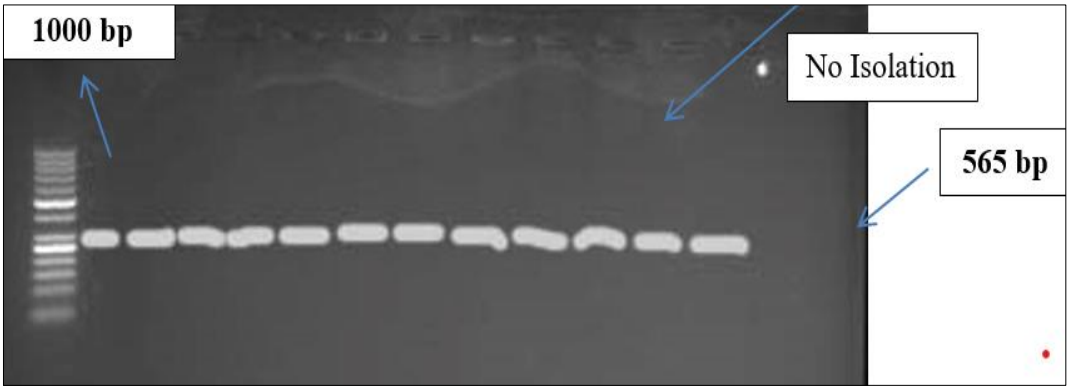

Fig 3: Gel electrophoresis of the Mrp amplicon. M: Marker, no. of the *P. mirabilis* isolates, Mrp amplicon size: 565 bp.

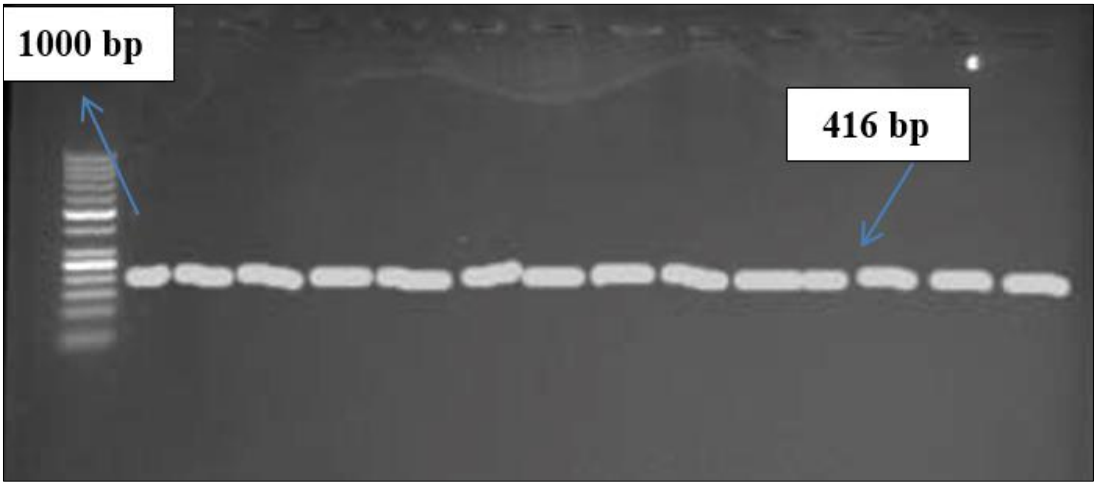

Fig 3: Gel electrophoresis of the MrkA amplicon product. From *P. mirabilis* isolates, amplicon size of the MrkA gene: 416 bp

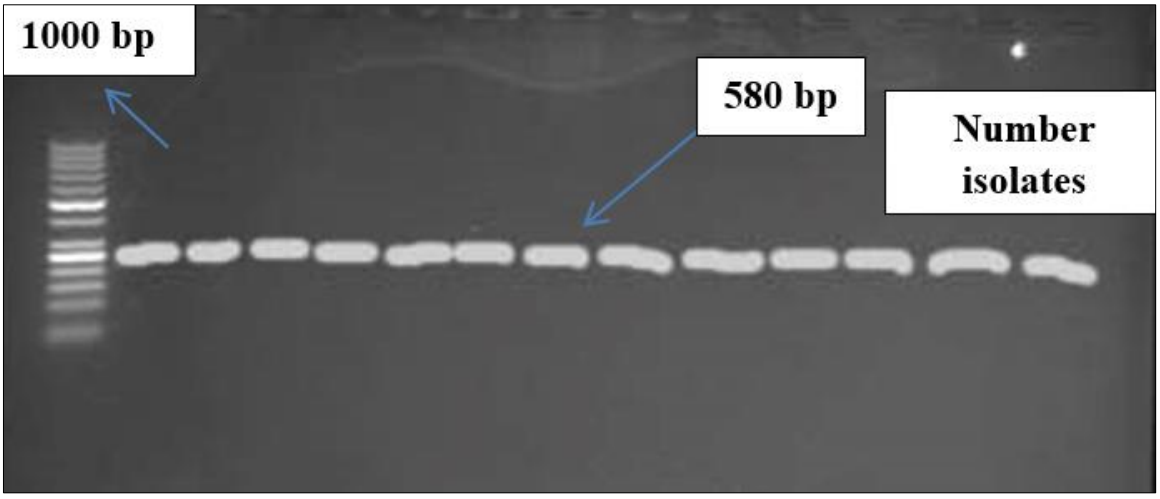

Fig 4: Gel electrophoresis of the Uca amplicon. M: Marker, No. From *P. mirabilis* isolates, Uca gene amplicon size: 580 bp

Inhibition of *P. mirabilis* reproduction by urea

On agar plates without urea, growth of *P. mirabilis* completely covered the plates; the diameter of the colonies grown on urea-containing dishes decreased with the increase in urea concentration, as shown in Figure 6.

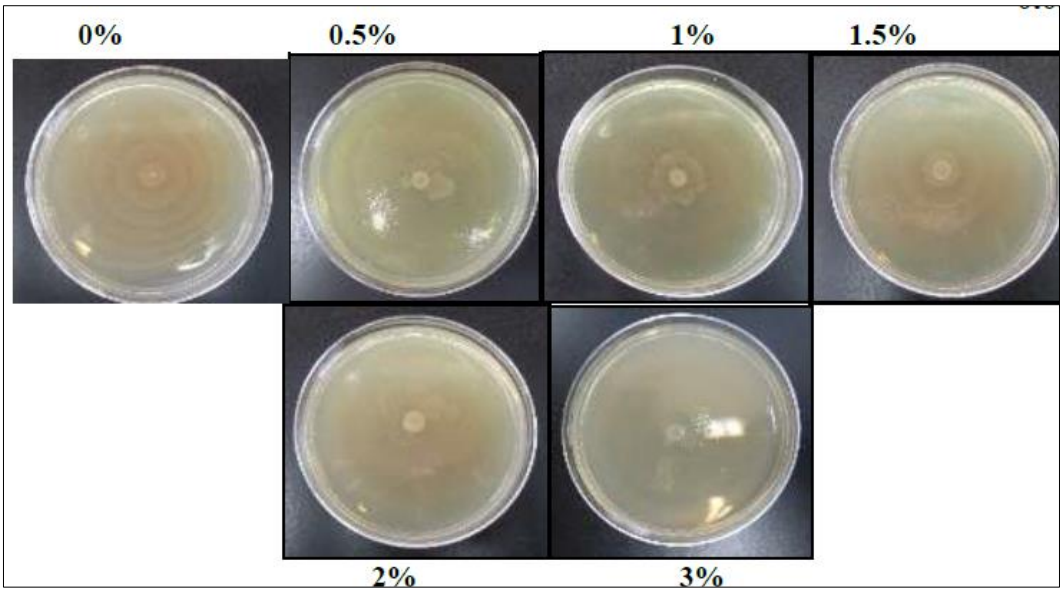

Fig 6: Halo images of swarming plates containing different concentrations of urea (concentration ratio, w/v).

More than one study confirming the anti-swarm property of urea in a solid medium, such as the study reported by Van Asten *et al*. (1999) who tested the utility of urea supplementation in isolating single colonies of *Staphylococcus intermedius*, and *Streptococcus spp.* and *P. aeruginosa* from mixed cultures with *P. mirabilis* by inhibiting the pooling of *P. mirabilis*, a study by Iwalokun *et al*. (2004) recorded the ability of urea at 0.75 - 1.25% to abstain from mobilizing clinical isolates of *Proteus* that allowed its identification.

Inhibition of *P. mirabilis* aggregation with ethanol and sodium azide

Other chemicals that have been shown to prevent swarming of *P. mirabilis* are ethanol and sodium azide. As shown in the Figure 7, there is a significant decrease in the swarming diameter of *P. mirabilis* in dishes containing (0.01% and 0.1%) sodium azide, and no swarming in another plate by adding two drops of (90%) ethanol.

Fig 8: Halo images of swarmed plates containing (0.01% and 0.1%) of more sodium azide and a plate are added to its cover two drops of ethanol 90%.

These results agree with other findings reported by Hernandez *et al.* (1999) and Al-Kaebi and Matrood (2012) who showed that ethanol and sodium azide are among the substances that have anti-sleak properties when added to a solid medium for isolating bacteria in mixed growth with *Proteus* strains; where they are considered among the substances that affect the activity of flagella. Chemical compounds that inhibit swarming are attributed to the flagella proteins of the swarming cells and cause their disintegration or impede flagella formation and motility. The enhanced or inhibited enhancement of *P. mirabilis* by all the chemical compounds tested in this study can be attributed to several reasons including: these compounds may act as extracellular or intracellular signals, and they may act as cytokinetic signals contacts that interact with some transmembrane sensor proteins or may affect membrane fluidity, these compounds may interact with the activity of RsbA proteins through an RsbA or RsbA-dependent independent pathway to regulate swarming and expression of virulence factor in *Proteus* or these compounds may have an inhibitory effect on the gene RsbA that regulates the swarming of *Proteus* (Liaw *et al*. 2004, Laftaah 2012).

Antibiotic sensitivity test for *P. Mirabilis*

In this study, we tested the effect of different antibiotics on *P. mirabilis* isolates. These isolates showed different sensitivity towards the antibiotics used in this study (Figure 9).

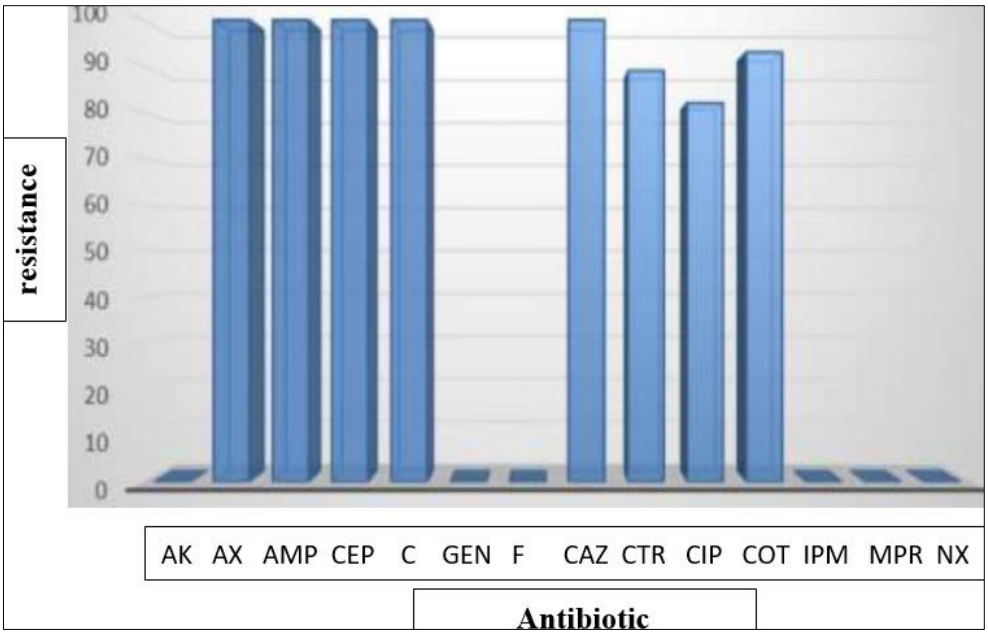

Fig 9: Antibiotic sensitivity of *P. mirabilis* (AK: Amikacin, AX: Amoxicillin, AMP: Ampicillin, CEP: Cephalothin, C: Chloramphenicol, GEN: Gentamycin, F: Nitrofurnation, CAZ: Ceftazidime, CTR: Ceftriaxone, CIP: Ciprofloxacin, COT: Cotrimazole, IPM: Imipenem, MPR: Meropenem, NX: Norfloxacin)

It was found that the majority of the isolates were multidrug resistant as they were resistant to three or more antimicrobials. The highest resistance was recorded with ampicillin (100%), amoxicillin (100%), cephalothin (100%), chloramphenicol (100%) and ceftazidime (100%), which are mild resistance cotrimazole 26/28 (92.8%), ceftriaxone 25/28 (89%) and ciprofloxacin 23/28 (82%) while all isolates were sensitive to amikacin, gentamycin, nitrofurante, imipenem, meropenem, and norfloxacin.

The emergence of multidrug-resistant strains may be that are resistant to most of the antimicrobials tested is due to the fact that ampicillin, amoxicillin and cephalotin are the most common antibiotics prescribed in hospital even before urine results, and also the most accessible. Ciprofloxacin resistance may be due to one of three mechanisms of resistance to quinolones: mutations that alter drug targets, mutations that reduce drug accumulation, and plasmid-mediated QNR genes that protect cells from the lethal effects of quinolones. These genes are mainly found in Enterobacteriaceae and influence the dynamics of development and acquisition of quinolone resistance (Hooper 2003, Fonseca *et al.* 2008).

On the other hand, all *P. mirabilis* isolates showed sensitivity to amikacin. This result is identical to that obtained by Okesola and Makanjuola (2009) and Al-Jumaa *et al*. (2011) sensitivity to norfloxacin (100%). Norfloxacin resistance is rare and if it does occur. In this case, the cause is a spontaneous mutation (range: 10-9 to 10-12 cells). Resistant organisms appeared during treatment with norfloxacin in less than 1% of treated patients (Padeiskaia 2003).In our study imipenem and meropenem, as carbapenems are effective antimicrobial drugs against Gram-negative bacteria and it is similar with reported by Sedlakova *et al*. (2011).

All isolates are resistant to ampicillin, amoxicillin, cephalotin, chloramphenicol and ceftazidime, and some isolates are resistant to ceftriaxone, ciprofloxacin and cotrimazole. While all isolates were sensitive for gentamicin, imipenem, meropenem, amikacin, nitrofurantion, and norfloxacin. The quorum was sensed by the production of homocerin lactone by bacterial isolates.

Conclusion

Identification of bacteria isolated in the clinical laboratory by sequencing or on a molecular basis rather than a phenotype that could improve clinical microbiology by better delineating a poorly labeled, rarely isolated, or strains chemically abnormal. The study of quorum sensing by other means such as thromone and some signaling proteins. Using active biological compounds from natural sources to inhibit the pathogenicity of bacteria. The use of the new antibiotic should be highly selective and not used for a while to reduce the chance of drug-resistant bacteria emerging.

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