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Molecular Detection of Some Virulence Factors of Uropathogenic *Proteus mirabilis*

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Abstract:
In the present study,120 urine samples were collected from patients suffering from urinary tract infection from both sexes and from different ages, during the period from July 2020 to November 2020 from the private laboratories in Hilla, Iraq, to detect *Proteus mirablis (P mirabilis)*bacteria, and the results of the biochemical tests and Vitek 2 system showed that 36(30%) samples belong to *P. mirablis*. Some of the virulence factors genes were investigated using Single Polymerase Chain Reaction Technique and they are *ureC* responsible for production of urease enzyme, *hpmA*, which is responsible for producing of hemolysin, *zapA* responsible for production of protease enzyme, *rsbA* responsible of regulatory of swarming phenomenon, *mrp* which responsible of mannose-resistant *proteus* like fimbriae, *flaA*, responsible for flagella, for the rates of the appearance of these genes were (100%), (100%), (100%), (100%),(80%) and (100%) respectively.

Keywords: Health sciences; Virulence factors; Patients
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Introduction:

Proteus mirabilis is considered an important infectious agent that is acquired both in the community and the hospital environment [1]. Urinary tract infections (UTI) caused by this microorganism can be divided into two categories, systemic or hematogenic, and ascendant that colonizes the whole urinary tract (from the urethra to the kidneys) which most frequently infects patients with long term urinary catheters or with anatomic anomalies in the urinary tract [2, 3].*Proteus mirabilis* is the third most common cause (after *Escherichia coli* and *Klebsiella pneumoniae*) of the UTI, but it is the most serious because it causes some clinical problems such as catheter obstruction, stone formation in the bladder and kidneys, cystitis, acute and chronic pyelonephritis, and bacteremia [4,5]. *P. mirabilis* infections are characterized as long term and difficult to treat and can often lead to death due to the capacity of this microorganism to mediate urea hydrolysis, via the urease it produces, causing tissue necrosis and inflammation at the infection site, so that antibiotics is not accessible to the pathogen[6]. Several pathogenicity factors and morphological characteristics are expressed by *P. mirabilis*, such as fimbria, flagellum, capsules, enzymes (urease, proteases and deaminase amino acid), toxins hemolysins, proteus toxic agglutinin (Pta), and endotoxins] that explain the difficulty in obtaining success in clinical therapy [7]. The hemolytic activity produced by *P. mirabilis* is associated to hemolysin *HpmA*. This hemolysin is associated to the cell, calcium-independent, former of pores, encoded by two genes, *hpmA* and *hpmB*, that regulate the *HpmA* (166 kDa) and *HpmB*(63 kDa) proteins, respectively. *HpmA* hemolysin is responsible for tissue damage and is activated when its N-terminal peptide is cleaved, resulting in active *HpmA*(140 kDa). HpmB is responsible for *HpmA* activation and transport [8]. Evidence suggests a correlation between the

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hemolytic activity and the invasive and cytotoxic capacity in the Vero cells (African green monkey kidney) by *P. mirabilis*, increasing the virulence of the infections. Swihartand Welch confirmed that hemolytic and cytotoxic activity are due to the production of active hemolysin*HpmA*, using mutant isolates (*HpmA* negative) that lose both the hemolytic and cytotoxic capacity in human cells (epithelial, monocyte) and in Vero cells. The pathogenesis of these bacteria is associated with possessing many virulence factors which include the pili (Fimbria), flagella, urease, protease, hemolysin and multi-sugars adipose (lipopolysacchrine), referred to as endotoxin[9].

Materials and methods:

A total of 120 urine samples were collected from patients with UTI. Samples were collected from patients during the period from July 2020 the end of November 2020 from the private laboratories in Hilla, Iraq.

Bacterial diagnosis: Isolation of *P.mirablis*bacteria was performed by a culture on both blood and MacConkey agar using and incubated aerobically at 37°C for 24 hours.Bacterial identification was done by biochemical test, namely indole, citrate, oxidase, catalase, urea hydrolysis,H_2S production, lactose fermentation also Vitek 2 system used for more identification[10].

Detection of heamolysin: it was performed according to Senior, and Hughes [11].

Detection of extracellular protease production: it was performed according to [12].

Detection of Colonization Factor Antigen: it was performed according to [13].

Genomic DNA Extraction: DNA was extracted of bacteria *P.mirablis* by using (Genomic DNA mini kit) processed from American company and according tosupplying company's instructions [14].

DNA electrophoresis in agarose gel:it was performed according to [14].

DNA Primers: The primers sequences and PCR conditions that used in study are listed in Table (1).

Contents of Reaction Mixture: Amplification of DNA done in final volume of (25µ) containing the following as outlined in Table (2). Detection of Amplified Products by Agarose Gel Electrophoresis Successful PCR amplification was confirmed by agarose gel/ electrophoresis by visualization against UV light [12]. Agarose gel was prepared. Then the comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5µl of DNA sample was transferred into each well of agarose gel, and in one well we put the 5µl DNA ladder. The electric current was allowed to pass at 70 volts for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera.

Table (1): The primers, sequences

Gene name	Primer sequence (5´- 3´)	Size of Bp	References
ureC	F:CCGGAACAGAAGTTGTCGCTGGA R:GGGCTCTCCTACCGACTTGATC	533	[15]
hpmA	F:GTTGAGGGGCGTTATCAAGAGTC R:GATAACTGTTTTGCCCTTTTGTGC	709	[16]
zapA	F:ACCGCAGGAAAACATATAGCCC R:GCGACTATCTTCCGCATAATCA	540	[17]
rsbA	F:TTGAAGGACGCGATCAGACC R :ACTCTGCTGTCCTGTGGGTA	467	[18]
mrp	F:ACACCTGCCCATATGGAAGATACTGGTACA R:AAGTGATGAAGCTTAGTGATGGTGATGGTGATGAGGTAAGTCACC	550	[19]
flaA	F:AGGATAAATGGCCACATTG R: CGGCATTGTTAATCGCTTTT	417	[20]

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Table (2): Polymerase Chain Reaction Mixture

No.	Mixture Contents	Volume (µl)
1.	Master Mix	12.5
2.	Forward Primer	2.5
3.	Reverse Primer	2.5
4.	Template DNA	5
5.	Nuclase -Free Water	2.5
Total		25

Results and Discussion

Isolation and diagnosis *P. mirablis*

The present study demonstrated 36 isolates belong to genus *P.mirablis* out of 120(30%) samples collected from patients with urinary tract infection, through the study of some cultural and microscopical characteristic and biochemical tests as follows. The colonies were single pale con MacConky agar, non-lactose fermenter as well as the smell of bacterial growth which is similar to smell of fish rotting and appeared ripple movement or swarming on the blood agar, which is the recipe initial diagnostic for this bacteria. Microscopic examination of the results showed that the bacterial cells isolate are short bacilli negative to Gram stain, non-spore forming. The results of biochemical tests were adopted as complementary characteristics of the initial diagnosis of *P. mirabilis*, where the results indicated that isolates belong to *P. mirabilis*, all isolates were oxidase and indol negative, catalase positive, and urease positive, but variable results were noticed about citrate utilization. The results of this study are identical with those obtained by [21] whohave been isolated *P. mirabilis* from cases of urinary tract infections. In addition, [22]and [23]also have been isolated *P. mirabilis*(27.1%),(29.2%) respectively, from patients with UTI.

Virulence factors of *P. mirabilis*

The phenotypic and genotypic characters were tested for all *P. mirabilis isolates* in this study in order for detection of the virulence factors. Thespecific primers were used for screening the presence of (*UreC, hpmA, ZapA, rsbA, mrp,* and *flaA*) genes. The results of the PCR for the isolates of *P. mirabilis* showed that allisolates were positive for the presence of *UreC* gene 36(100%) (Figure-1).

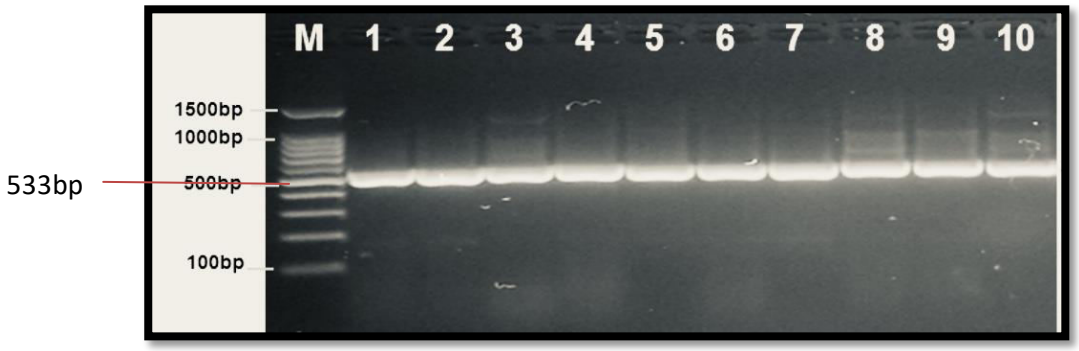

Figure (1): agarose gel electrophoresis (1.5%) of RCR amplified of *Urec* gene (533)bp of *P.mirabilis* for (55)min at (70) volt L: DNA molecular size marker (100-1500 bp ladder).

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All isolates of *P. mirabilis* were phenotypically positive (100%) for extra cellular urease. The results are in agreement with the results of [24,25], who found that (100%) of *P. mirabilis* isolates showed strong production of urease. Urease is one of the most important factors in *P. mirabilis* pathogenesis. *In vitro* (on basic urea agar), urease hydrolyzing urea to alkaline ammoniaand carbon dioxide, thereby increasing the pH and will be changing the color of phenol red indicator to pink [26]. But *In vivo* (human body) this enzyme catalyzes the formation of kidney and bladder stones or to encrust or obstructs indwelling urinary [27]. The urea-inducible urease gene cluster (ureRDABCEFG) encodes a multimeric nickel-metalloenzyme that hydrolyzing urea to alkaline ammonia and carbon dioxide, thereby increasing the pH and facilitating the precipitation of polyvalent ions in urine resulting in stone formation. This pH alteration is important during *P. mirabilis* catheter colonization, facilitating the bacterial adherence and formation of biofilm. Urease activityincreased in the swarmer cells and is necessary for a *P. mirabilis* infection, while swarmer cells up-regulates the production of urease, swarming is not needed to form stones or crystalline biofilms [28]. Lastly, stone formation is considered a hallmark of *P. mirabilis* infection, providing a number of advantages for these bacteria such as: protection from the host immune system, the host immune system protection, blockage of the ureters, ammonia toxicity to host cells and direct tissue damage. These can lead to formation of protective and nutrient-rich environmental niche for these microorganisms [29].*P. mirabilis* isolates were evaluated to confirm their ability to produce extracellular hemolysin on blood agar, it was found that 100% of isolates have the ability to produce extracellular hemolysin. These results in agreement with the results of [30, 31, 32 and 33]who demonstrated that all *proteus* isolates produce hemolysin on blood agar plates. The function of hemolysin is to form pores in target host cells. It was proposed that hemolytic activity helps *P. mirabilis* to spread into the kidneys during infection. This is probably mediated through the increased ability of hemolytic *P. mirabilis* cells to invade host tissue [34].*hpmA* gene which responsible for producing hemolysin and considered as important virulence factor for *P.mirablis*. In this study, *hpmA w*as been, and the results showed that 36 isolates in a rate of (100%) had *hpmA* gene as shown in (Figure 2),and this result is similar to [35]as they reported that the rate of this gene in *P. mirablis* isolates was (97.15%).The hemolysin enzyme acts on destroying the leukocyte membrane by making small holes in the membrane of the leukocyte and epithelial cell and its presence is a very important factor in providing the bacteria with iron; and because of its cytotoxic, it leads to the destruction of the kidney tissue of the host[36].

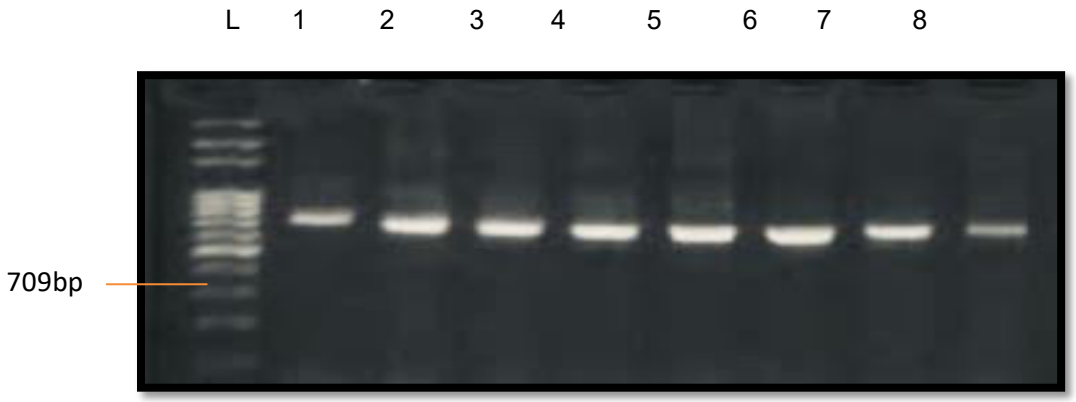

Figure (2): agarose gel electrophoresis (1.5%) of RCR amplified of *HmpA* gene (709) bp of *P.mirabilis* for (55) min at (70) volt L: DNA molecular size marker (100-1500 bp ladder).

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The ability of *P. mirabilis* to produce extracellular protease in M_9media (supported by 20% glucose and 1% gelatin) was investigated and it wasfound that all of the study isolates 36(100%) were able to produce extracellular protease after 24 hours of incubation which was indicated by the presence of a clear hallo of transparent area around the colony after the addition of 3 ml of trichloroacetic acid (5%). Also, the presence of *ZapA*gene has been tested for all isolates which gave positive results in all 36 (100%) isolates (Figure 3). These results were similar to the results obtained by [37, 38] which showed that100% of *P. mirabilis* produced protease. Metalloproteases are a major group of proteolytic enzymes in *Proteus*isolates. The protease produced by *P. mirabilis* is a metalloprotease of serralysin family of zinc proteases, encoded by *zapA* gene.These proteases which have the capacity to degrade host proteins releasing amino acid as nutrients and may degrade proteins such as IgA which are involved in host defense and may also be involved in host tissue damage. *P. mirabilis* that lost IgA protease activity, has normal urease production activity, hemolytic activity, swarmer cell differentiation, production of flagella, swarming motility, and biofilm formation [39].

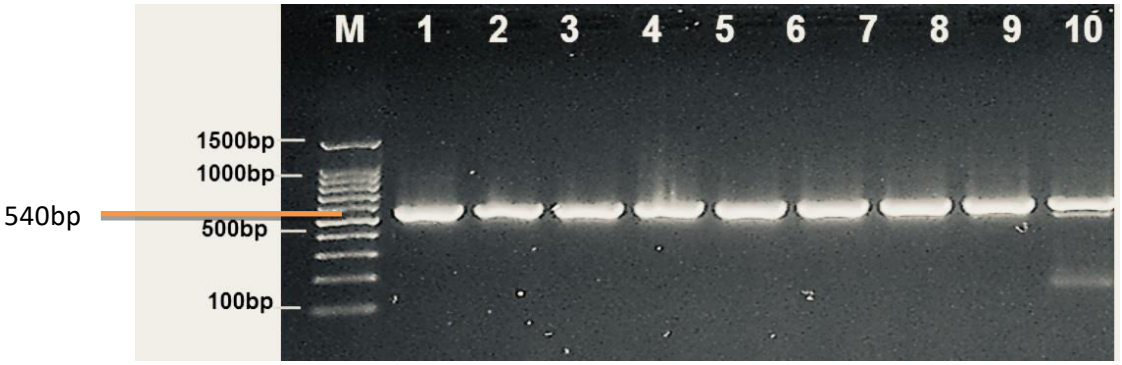

Figure (3): agarose gel electrophoresis (1.5%) of RCR amplified of *zapA* gene (540) bp of *P.mirabilis* for (55) min at (70) volt L: DNA molecular size marker (100-1500 bp ladder).

In this study, *rsbA* gene was detected as shown in figure (4),and the results showed that 28(80%)were positive which is consistent with the previous studies [40, 41]. In the current study, a phenotypic character of swarming, the bull's-eye ring was exhibited by all the isolates. Nevertheless, swarming regulated genes are not necessarily required for swarming, for the reason that so many genes andoperons are involved in the process [42].

Fimbriae are bacterial surface appendages used for adherence. Phenotypically, the results showed that all isolates (100%) agglutinate human red blood cells type A in the presence of mannose or tannic acid which indicates that all *P. mirabilis* isolates have CFA I and CFA III.This finding is in agreement with other studies such as [43] who found that (100%) *Proteus* isolates were carrying colonization factor antigen (CFA)type (I, III).Results amplification of region of the *mrpA*gene showed that all isolates 36(100%) were carrying this gene (Figure 5).These results were similar to the results obtained by [44, 45] which reported that (100%) of *P. mirabilis* and *P. vulgaris* respectively, carrying this gene. Likewise, these results were similar to that results reported by [46] who found (95.8%) *P. mirabilis* isolates gave positive result to CFA in the presence of D-mannose. MR/P fimbriae are encoded by *mrp* operon containing 10 genes located on bacterial chromosome. The fimbrial genes are transcribed and MR/P fimbriae are synthesized when the promoter is in on orientation [47].

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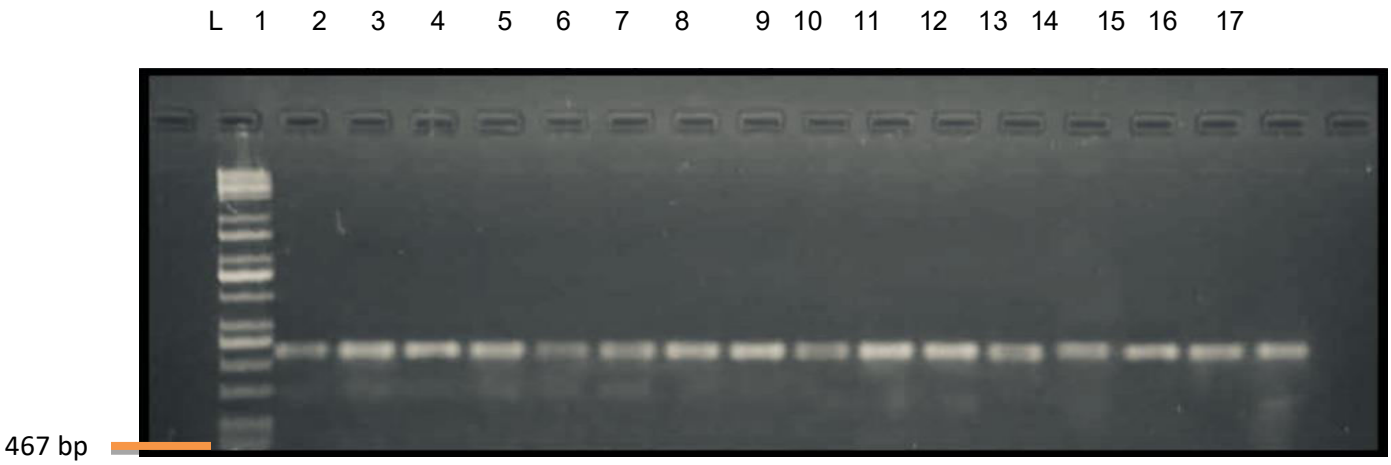

Figure (4): agarose gel electrophoresis (1.5%) of RCR amplified of *rsb* gene (467) bp of *P.mirabilis* for (55) min at (70) volt L: DNA molecular size marker (100-1500 bp ladder)

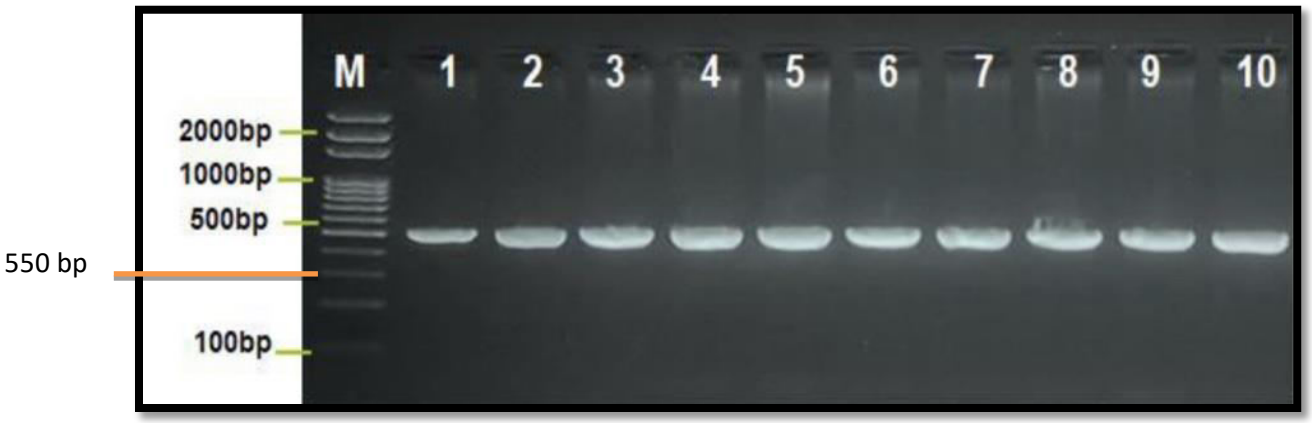

Figure (5): agarose gel electrophoresis (1.5%) of RCR amplified of *mrp* gene (550) bp of *P.mirabilis* for (55) min at (70) volt L: DNA molecular size marker (100-1500 bp ladder)

The molecular method was used to detect *flaA* gene. The results of this study revealed that all isolates 36(100%) possessed *flaA* gene (Figure 6). This finding is in agreement with other researches [48]. They reported the presence of *flaA* gene in *Proteus* isolates (100%). The biosynthesis of flagella is a key process in both motility and swarming and involves numerous genes on the *proteus* chromosome. The filamentous portion of P. *mirabilis* flagellum is mainly composed of the flagellin protein FlaA, encoded by *flaA*.There are three copies of flagellin-determinant gene (*flaA*, *flaB*, *flaC*) that reside on the *P. mirabilis* genome with only one copy that is actively expressed [49].Considering that flagellin is strongly antigenic, it has been postulated that this recombination could contribute to immune evasion during infection [50].

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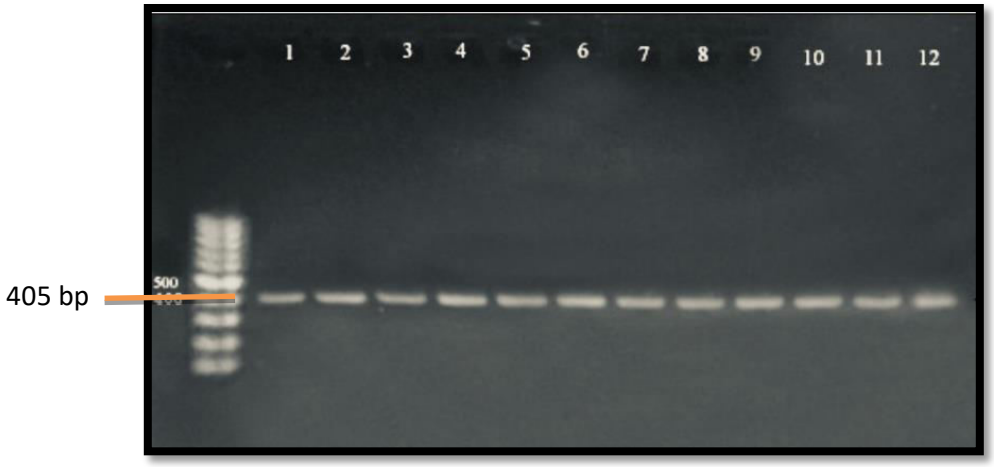

Figure (6): agarose gel electrophoresis (1.5%) of RCR amplified of *flag* gene (405) bp of *P.mirabilis* for (55) min at (70) volt L: DNA molecular size marker (100-1500 bp ladder)

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