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RESEARCH PAPER

Molecular characterization and antibiotic susceptibility of *Proteus mirabilis* isolated from different clinical specimens in Zakho city, Kurdistan Region, Iraq.

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A B S T R A C T:

Proteus mirabilis is one of the important causative agents of bacterial infections in humans. This study involved the prevalence of the virulence genes among *P. mirabilis* from urine, ear, sputum, burn, wound, and vagina specimens in Zakho city during the period of July 2021 until January 2022 and their susceptibility to different commonly used antimicrobial agents. Isolates were identified by traditional phenotypic and biochemical tests. Out of 400 cultures, 95 (23.75%) were *P. mirabilis*. The antibiotic susceptibility toward different antibiotics varied among the isolates. The results showed that ceftriaxone was the most potent antibiotic with a susceptibility rate of 90.28 %. The isolates were resistant to many screened antibiotics, with the highest rate of 88.42% to imipenem. Whereas, the resistant proportion was slightly lower toward other antibiotics at rates varied from 74.68 % and 77.89% for Ampicillin and Amoxicillin/clavulanic acid, respectively. Fifty-two *P. mirabilis* isolates were selected for PCR analysis, according to their multiple antimicrobial resistance to the used antibiotics. The selected samples were amplified for *P. mirabilis* identification by producing a single band of the *ureR* gene. The prevalence of the virulence genes (*flaA, rsbA, zapA, and mrpA*) among these isolates were 96.15%, 88.46%, 80.77%, and 69.23%, respectively. This study demonstrates that multidrug resistance *P. mirabilis* harbors multiple virulence genes.

KEY WORDS: *P. mirabilis*; Virulence genes; Antibiotics resistant
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1.INTRODUCTION:

Proteus mirabilis is one of the most remarkable *Proteus* species that belongs to Enterobacteriaceae. *P. mirabilis* has a cosmopolitan distribution since it can be isolated from soil, water, animals, and humans (Gorički, et al., 2017). In hospitals, these bacteria are responsible for about 90% of Proteus spp. infections (Ramos et al., 2018). *Proteus* spp. has various methods of transmission and consequently could cause infection in numerous anatomical areas of the body especially the urinary tract, ear, respiratory tract, skin, wounds, burns, vagina, and gastrointestinal tract (Abd Al-Mayahi, 2017).

The drug-resistant capacity among *P. mirabilis* strains has been increased recently and this is a major clinical problem for treatment (Majeed et al., 2019). These bacteria have developed antibiotic-resistance strategies by the formation of new genes transferred through plasmids and other mobile genetic elements such as transposon and enteron (Evans et al., 2020). The outcomes of these processes have revealed several strains with multi-drug resistance patterns. These strains use multiple approaches in acquiring multidrug resistance such as changing the target site of antibiotics, deactivating the antibiotics by enzymes, efflux mechanisms for removing drugs, and increasing mutation rate as a consequence of stress response (Bameri et al., 2018). Many studies showed alarming results about antibiotic-

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resistant of these bacteria, for example, Alabi et al. (2017) illustrated that 55% of *P. mirabilis* were multi-drug resistant. Another study in Kurdistan by Naqid et al. (2020) has reported elevated results about multidrug-resistant *P. mirabilis*. Nevertheless, within healthcare facilities, the prevalence of drug-resistant *P. mirabilis* was close to that of *E. coli* (38% to 48.5%) (Girlich et al., 2020).

Proteus mirabilis has developed various virulence determinants that help it to grow and survive in the host body. These virulence factors are associated with disease-producing potentials including; urease production (*ureR*), flagella (*flaA*), fimbriae(*mrpA*), protease enzyme production (*zapA),* swarming regulator gene (*rsbA)* (Abd Al-Mayahi, 2017). The urease gene cluster which comprises ureRDABCEFG, produces a cytoplasmic nickel metalloenzyme that is regulated by *ureR* gene which is used for the identification of *P. mirabilis* and enhances stone formation in kidney and bladder (Milo et al., 2021). Flagella, which is encoded by *Proteus* chromosome *flaA* gene, is crucial for motility and swarming phenomenon, also has been postulated that it may participate in immune evasion during infection (Milo et al., 2021). The protease enzyme encoded by *zapA* gene seems to be able to degrade host protein and lead to tissue damage (Norsworthy et al., 2017). Quorum sensing *rsbA* gene facilitates biofilm formation and regulates the swarming motility, which encodes a sensory and acts as a protein sensor in the environment (Hussein et al., 2020). Mannose-resistant/Proteus-like fimbriae (MR/P) which are encoded by *mrpA* gene are required for the early stages of infection, including clusters formation, colonization, and evasion from the host immune response (Ghaima et al., 2017). Due to limited studies on virulence genes this bacterium, since there are limited studies which was performed on this bacterium in Zakho city for example, Jameel and Artoshi (2019) demonstrated the prevalence of *P. mirabilis* among urinary tract infections in diabetics and non-diabetics patients without investigating the molecular characteristics of the isolates involved in infection. Another study in Erbil city by Kamil and Jarjes (2021) investigated the distribution of a single gene (*ureR*) which is responsible for *P. mirabilis* identification without detecting the virulence genes. Therefore, the present study was adopted to illustrate the epidemiology of virulence genes of this pathogen among different clinical samples. Furthermore, to investigate their susceptibility to different antibiotics prescribed to patients by physicians to evaluate their effects and to detect the virulence-related gene; *mrpA, flaA, rsbA, zapA* using species-specific primer for identification of *P. mirabilis.*

2.MATERIALS AND METHODS

2.1. MATERIALS

2.1.1 Collecting specimens:

Four hundred specimens were collected from symptomatic out-patients suffering from urinary tract infections (150), external otitis media (90), respiratory tract infections (62), burns (23), wounds (30), and vaginitis (45) from out-patients who visited hospitals and private clinics in Zakho city from July 2021 to January 2022. For identification purposes, each specimen was cultured on different labeled culture media and then was identified by phenotypic characteristics including swarming motility on blood agar, non-lactose fermenting on MacConkey agar, and urease production in urea agar medium. Furthermore, catalase, motility test, oxidase, triple sugar iron agar medium test was done to differentiate *P. mirabilis* from other suspected microbes on the same medium.

1.2. Identification of Proteus mirabilis

On MacConkey agar *P. mirabilis* forms pale, non-lactose fermented, and yellowish colonies. On blood agar, no colonies were formed instead *P. mirabilis* isolates showed the swarming phenomenon with a fishy odor. The biochemical test results of *P. mirabilis* are shown in table (1).

Table 1: Conventional biochemical tests for identification of *Proteus mirabilis*

Test	Results
Urease	+
TSI agar	K/A*
Indole test	-
Motility	+
Citrate Utilization	-
Catalase test	+
Oxidase test	-
lactose Fermentation	-

(+) a positive result, (-) negative result. * K/A= glucose fermented with H2S precipitate and gas production.

1.3. Antibiotic susceptibility test

Fourteen different antibiotic discs were selected based on CLSI and physician prescription for treatment. The antimicrobial susceptibility of *P. mirabilis* was determined using disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines for the antimicrobial agents represented in table (2).

Table 2: Antibiotic discs

Antibiotic disc	Concentration (µg) Bioanalyse (Turkey)
Amikacin	10 µg
Amoxicillin	25 µg
Amoxicillin/clavulanic acid	20/10 µg
Ampicillin	20 µg
Cefotaxime	30 µg
Ceftriaxone	10 µg
Chloramphenicol	10 µg
Ciprofloxacin	10 µg
Cefixime	5 µg
Gentamicin	10 µg
Imipenem	10µg
Nalidixic acid	30 µg
Trimethoprim	10µg
Trimethoprim/ Sulfamethoxazol	1.25/23.75 µg

1.4 DNA extraction for identifying the virulence genes

Bacterial DNA was extracted from 52 isolates depending on their resistance rate by using a commercial Genomic DNA Mini extraction kit (Favorgen/Taiwan), according to the instructions supplied by the company. Following genomic extraction, the NanoDrop Spectrophotometer (Thermo Fisher Scientific) was used to measure the concentration and purity of the samples, and then PCR amplification was done.

1.5. Detection of *Proteus mirabilis* using species-specific PCR

In this study, five primers were used as mentioned in table (3). A species-specific primer *ureR* was used for detecting *P. mirabilis*, which is also responsible for the production of the urease enzyme. The four other primers *mrpA, flaA, zapA,* and *rsbA,* respectively, were used for detecting the four virulence-related genes including; fimbriae, swarming- flagellin, extracellular protease, and the regulator of swarming behavior.

Table 3: primers size and sequence of *Proteus mirabilis* used in this study.

Primers	Sequences	Size (bp)	References
ureR	**F-GGTGAGATTTGTATTAATGG** **R-ATAATCTGGAAGATGACGAG**	225bp	(Zhang et al., 2013)
mrpA	**F- ACACCTGCCCATATGGAAGATACTGGTACA** **R- AAGTGATGAAGCTTAGTGATGGTGATGGTGATGAGGTAAGTCACC**	550bp	(Zunino et al., 2001)
flaA	**F-AGGATAAATGGCCACATTG** **R- CGGCATTGTTAATCGCTTTT**	417bp	(Ali and Yousif, 2015)
rsbA	**F-TTGAAGGACGCGATCAGACC** **R-ACTCTGCTGTCCTGTGGGTA**	467 bp	(Badi et al., 2014)
zapA	**F-ACCGCAGGAAAACATATAGCCC** **R-GCGACTATCTTCCGCATAATCA**	540 bp	(Stankowska et al., 2008)

1.6. Detection of virulence genes by Polymerase Chain Reaction

The primers sequences used to amplify genes encoding virulence genes are listed in table

(3). The final volume of 20µl was prepared that contained 10µl of the master mix, 1µl of each forward and reverse primers (10 pmol/µl), 2µl of DNA genome (30-70 ng/µl), and 6 µl of PCR di-ionized water. The conditions of PCR amplification are shown in table (4). Beyond amplification, the amplicons were run on 1.2% (w/v) gel electrophoresis using 1.2 g of agarose prepared in 1x Tris-Boric-EDTA (TBE) buffer. The conditions of electrophoresis were 45V for 15 minutes then changed to 80V for 50 minutes. After running, DNA bands were visualized using a U.V. transilluminator (Cleaver scientific) light source (Yılmaz and Gok, 2012).

Table 4: PCR conditions for *P. mirabilis* species-specific primer and virulence genes.

Gene name	Temperature (c)/Time						
	Initial denaturation	Cycling conditions			Final extension	Cycle no.	Reference
		denaturation	annealing	Extension			
ureR	94 ^{0}C/4min.	94 ^{0}C/40sec.	58 ^{0}C/1 min.	72^{0}C/20sec.	72^{0}C/4min	40	(Zhang et al., 2013)
mrpA	94^{0}C/3min.	94^{0}C/1min.	40^{0}C/1min.	72^{0}C/1min.	72^{0}C/5min	30	(Zunino et al., 2001)
flaA	93^{0}C/3min.	95^{0}C/30sec	54.2^{0}C/30sec	72^{0}C/30sec.	72^{0}C/5min	30	(Ali and Yousif, 2015)
rsbA	94^{0}C/5min.	94^{0}C/1min.	58^{0}C/48sec.	72^{0}C/1min.	72^{0}C/7min	35	(Badi et al., 2014)
zapA	95^{0}C/1min.	94^{0}C/30sec.	53^{0}C/1min.	72^{0}C/1min.	72^{0}C/5min	35	(Stankowska et al., 2008)

2. RESULT AND DISCUSION

2.1. Prevalence of *Proteus mirabilis*

Out of 400 different clinical specimens, (95) 23.75% of *P. mirabilis* were isolated. The highest prevalence of (42/150) 28% was found in urine specimens, followed by nearly equal rates of 24.44% (22/90) from the ear and (5/30) 23.33% from wounds. On the other hand, *P. mirabilis* was isolated at rates of (4/23)17.34%, (13/62) 20.97%, and (9/45) 18%, respectively from burns, sputum, and vagina as shown in table (5). In this study higher rate of positive isolates were recorded in urine samples, because the number of urine samples were high as compared with other sources. Similar findings were reported by Gomaa *et al.* (2019), who showed that urine had the highest rate of *P. mirabilis* among other clinical sources. This is usually linked to the possession of many virulence factors which are significant for causing urinary tract infections, these virulence factors include adherence capability, urease production, and flagella (Jarjes, 2019). Another study in Baghdad city by Al-Bassam and and Al-Kazaz (2013) showed similar findings. Whereases, the significant proportion of isolation that was collected from ear swabs may be due to the influence of several factors that led to ear infection, including infection of the upper respiratory tract with viral infections that led to blockage of the Eustachian tube and thus fluid collects inside the ear, and any small wound due to the excessive cleaning will facilitate for bacterial growth in the ear (Alabi et al., 2017). The high prevalence of *P. mirabilis* in wound and burn are linked to the exposed area to microbial invasion, as a result of the necessary methods in terms of cleanliness, and poor sterilization rules (Perween et al., 2015).

Table 5: Prevalence of *Proteus mirabilis* from different sources.

Isolation Source	Number of Specimens	NO. of *Proteus mirabilis* Isolates	% Of *P. mirabilis* from each isolated source
Urine	150	42	28.00
Ear	90	22	24.44

Wound	30	5	23.33
Burn	23	4	17.34
Sputum	62	13	20.97
Vagina	45	9	18.00
Total	400	95	23.75

2.2. Antibiotics sensitivity test

The results of antibiotic susceptibility showed that the isolates vary in their sensitivity and resistance to the used investigated antibiotics as shown in table (6). The 3rd generation cephalosporins groups antibiotics which include Ceftriaxone, Cefotaxime, and Cefixime were the most potent antibiotics against *P. mirabilis*. The third generation of cephalosporins These antibiotics are usually more potent against Gram-negative bacteria than the first and second generations (Zanichelli et al., 2019). The potency of these drugs is attributed to the long half-life of the drug which leads to substantial usability and financial advantages (Thabit et al., 2020). The same finding was recorded in Jordan by Hussein et al. (2020) who showed that low rate of resistance of *P. mirabilis* toward the 3rd generation of Cephalosporins among urine samples. Likewise, Tabatabaei et al. (2021) in Iran found high potency of this group of antibiotics in catheterized urinary tract patients. On the other hand, the present results contradict the findings of Ahmed (2015) in Baghdad city who found that *Proteus* species isolated from the burn, wound, and urine samples displayed high resistance to this group.Imipenem had the highest resistance rate (88.42%) compared to other used antibiotics. Therefore, is not effective in the treatment of infection with this bacterial species. The high level of resistance to Imipenem in the isolated *P. mirabilis* could be due to many reasons such as the loss of porins, reduced expression of penicillin-binding proteins (PBPs) PBP1a, PBP2, or horizontal acquisition of various antibiotic resistance genes, including carbapenemase genes (Girlich et al., 2020). In Tikrit city, Al-Jebouri and Al- Alwani (2015) recorded a high resistant rate to Imipenem among renal failure patients. Additionally, in Iran Tabatabaei et al. (2021) in their study on catheter-associated urinary tract infections confirmed that *P. mirabilis* isolates are resistant to Imipenem, and they attributed it to the possibility of abusing antibiotics intake that exerted selected pressure on the emergence of multiple-resistant bacteria in community.The resistant proportion toward Trimethoprim and Trimethoprim/ sulfamethoxazole were also elevated which accounted for 65.26% and 67.37%, respectively. Resistance to these antibiotics is mediated through barrier permeability exhibited by isolated strains (Girlich et al., 2020). Furthermore, the resistance rate was elevated toward the penicillin group including; Amoxicillin/ Clavulanic acid, Amoxicillin, and ampicillin with rates of 77.89%, 64.21%, and 74.68%, respectively. Thabit et al. (2020) attributed the increasing resistance of the penicillin group to the elevated level of β-lactamase production as directly proportional to an increase in the resistance menace and frequent prescribing of this drug by physicians. The resistance of *P. mirabilis* toward the antibiotics used in this study was somewhat similar to other studies performed in Kurdistan Region /Iraq and other developing countries. For example, in Zakho city Jameel and Artoshi (2019) showed that these bacteria displayed low sensitivity rates to Chloramphenicol 14.29% and Amikacin 28.57%. Naqid et al. (2020) in Duhok city demonstrated that *P. mirabilis* displays high resistance to Ampicillin 77.8%, but low resistance to Gentamicin 11.1%. Similarly, Agha and Al-Delaimi (2021) in Duhok city found a high susceptibility of this bacterium to Gentamycin and Ciprofloxacin which was accounted for approximately 65%. In Diwaniyah city, Jawad and Alramahy (2017) reported variable resistance to Ampicillin 84.05%, Cefotaxime 53.62%. Raheem *et al.* (2019) which was in Baghdad city found the resistance rate for Ampicillin and Ciprofloxacin was nearly 60 %. Furthermore, Hussein et al., (2020) in Jordan showed that the isolated *P. mirabilis* showed moderate resistance to Sulfamethoxazole at 55.6%. Drug resistance to Proteus isolates might be due to the outer cytoplasmic membrane which contains lipoproteins, lipid bilayer, and lipopolysaccharide (Aghapour et al., 2019). Furthermore, the resistance of *Proteus* species has been linked with inappropriate usage of antibiotics (insufficient therapy or abuse in the use of antibiotics for treating patients)

therefore, it is mandatory to implement stringent rules for distributing antibiotics in the community to forbid the emergence of increasingly resistant isolates of harmful bacteria. Moreover, physicians must depend on laboratory counseling, in the diagnosis of infectious cases before prescribing any antibiotic to the patient (Korytny et al., 2016).

Another crucial mechanism that participates in the increase in the resistance of antimicrobial agents to infections is the introduction and clonal expansion of competitive resistant strains of *P. mirabilis* in the community (Girlich et al., 2020).

Table 6: Antibiotic susceptibility patterns of *P. mirabilis.*

Antibiotics	Code	Resistance		Sensitivity	
		No.	%	No.	%
Ampicilin	AM	70	74.68	25	26.31
Amoxicillin	AX	61	64.21	34	35.76
Amikacin	AK	28	29.47	67	70.53
Amoxicillin/ clavulanic acid	AM C	74	77.89	21	22.11
Cefixime	CFM	36	37.89	59	62.1
Chloramphenicol	C	54	56.84	41	43.16
Ciprofloxacin	CIP	39	41.05	56	58.95
Cefotaxime	CTX	25	26.32	70	73.68
Ceftriaxone	CRO	9	9.47	86	90.2
Gentamicin	CN	44	46.32	51	53.68
Imipenem	IMP	84	88.42	11	11.58
Nalidixic acid	NA	45	47.37	50	52.63
Trimethoprim/ sulfamethoxazole	SXT	64	67.37	31	32.63
Trimethoprim	TMP	62	65.26	33	34.74

Overall No. Tested	**95 *P. mirabilis***

2.3. Molecular Analysis

Out of 95 positive specimens, 52 isolates were selected for molecular identification depending on their resistance rates to the 14 used antibiotics. The results illustrated that all isolates have amplified a species-specific region via producing a single band of *ureR* gene of 225 bp as shown in figure (1). The *ureR* gene is a crucial virulence factor for the genus Proteus, which also acts as a transcriptional activator of the urease gene and can regulate its transcription. Studies have confirmed the presence of urease in *P. mirabilis,* which permits these species for rapid acclimatization and proliferation in the digestive tract at a pH environment of 5–10 (Abdullah et al., 2017). The *ureR* gene is one of the most widely accepted target genes for the identification of *P. mirabilis* (Liu et al., 2019; Wang et al., 2019). The amplification of the *ureR* gene in all isolates confirmed that they were *P. mirabilis.* Similarly, Kamil and Jarjes (2021) in Erbil using the same primer for the identification of *P. mirabilis* also obtained the same segment size (225bps). Many other researchers used the *ureR*-based method for the identification of *P. mirabilis* such as Zhang *et al*. (2013) in China who designed a species-specific primer based on *the ureR* conserved region of *P. mirabilis* for identification purposes by PCR.

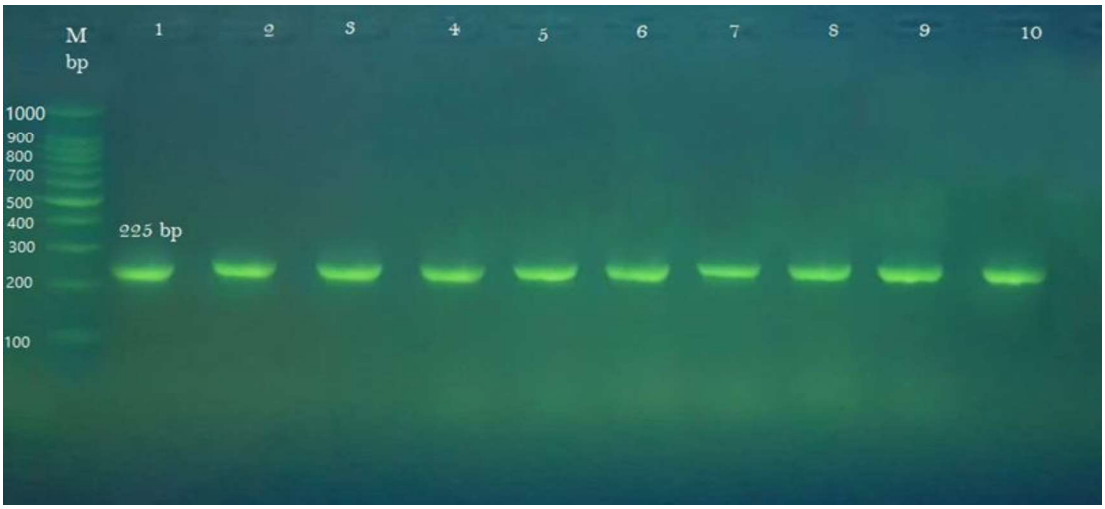

Figure 1: The amplification of PCR for *P. mirabilis* identification: Using specific-species *ureR* primer with molecular weight 225 bp. The Lane M contained a DNA ladder of 1000-100bp molecular weigh

Proteus mirabilis that possessed *ureR* gene were subjected to molecular methods for investigating the distribution of the virulence genes among patients in Zakho city, which includes; *mrpA, flaA, rsbA, zapA* as shown in figure (2). *flaA* gene was the most common virulence gene among *P. mirabilis* isolates which appeared in 96.15% (50/52). The flagella that are encoded by *flaA* gene are recognized as one of the major virulence factors that assist these bacteria to move aggressively (Jawad et al., 2017).

The second most common virulence gene was *rsbaA* gene which was detected in 46 isolates

in total with a rate of 88.46%. In this study, a phenotypic character of swarming was detected in all isolates. However, swarming regulated genes are not essential for swarming, since many genes and operons are involved in the process (Naseri et al., 2018). Whereas, the prevalence of *zapA* was 80.77%. Ali and Yousif (2015) stated that *zapA* can degrade many proteins *in vivo* such as IgA and IgG antibodies, thus minimizing the immune response, and for this

reason, is considered an important virulence factor for this pathogen. *mrpA* gene is important for adherence and biofilm formation had a prevalence of 69.23%.These results are somewhat following a study in Baghdad, which illustrated the occurrence of virulence-related genes *rsbA* and *mrpA* at rates of 100% and 80%, respectively (Al-Hamdani and Al-Hashimy, 2020). Another study in Iraq by AL-Oqaili et al. (2017) reported the prevalence of *mrpA* and *flaA* among *P. mirabilis* at rates of 40% and 100%, respectively. On the other hand, Ram et al. (2019) found that the distribution of *flaA* gene was only 28.5% which contradicts the finding of this study. Furthermore, the occurrence of *zapA* gene was somewhat similar to the results obtained by Abd Al-Mayahi (2017), who stated that 100% of *P. mirabilis* strains possess *zapA* gene.

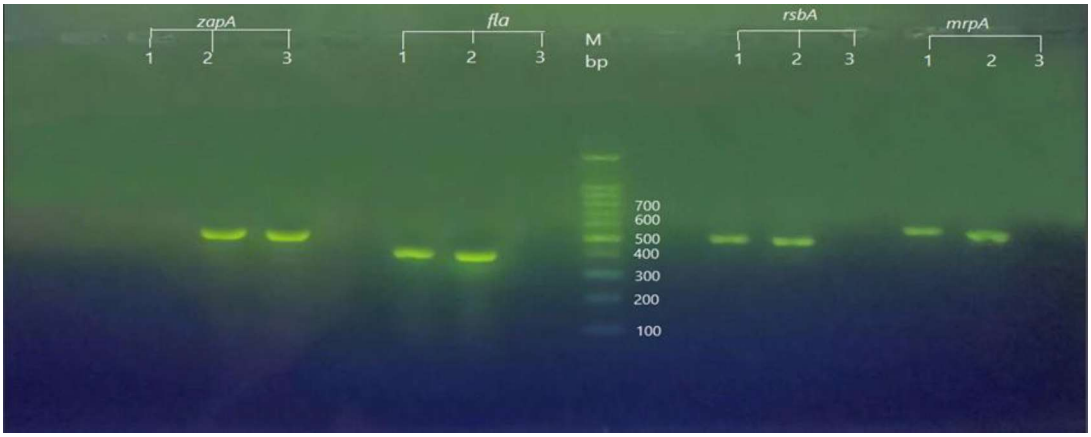

Figure 2: The PCR amplification of four virulence gene using four primers (flaA, rsbA, mrpA, zapA) with variant amplicon size. Lane M represents a DNA marker of 1000-100bp.

The reported variation in the current study among virulence genes could be due to specimen size, environmental conditions of each region and the distribution of virulence genes among different populations (Tolulope et al., 2021).

3. CONCLUSION

Proteus mirabilis was the most isolated organism especially in urine samples specimens than other clinical sources used possessing variant virulence genes in addition to showing antimicrobial resistance which is an alarming condition as these isolates possess a variant virulence and multiple antimicrobial resistance characteristics. The 3rd generation cephalosporins antibiotic showed the highest efficiency against this bacterium in vitro, therefore, they should be recommended by physicians for the treatment of P. mirabilis. Additionally, the primer ureR gave effective identification of the tested isolates. It is worthwhile, to mention that diverse virulence factors assist the spreading of microorganisms in various clinical sources.

Conflict of Interest: there is no conflict of interest.

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