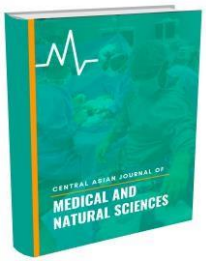

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Article

Molecular Identification of Several Genes in Isolates from Vaginal Secretions Linked to Biofilm Formation and Antibiotic Resistance

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Abstract: Bacterial vaginitis, which affects millions of women worldwide, is linked to numerous major health issues, and cause by numerous aerobic and facultative bacteria including, *Klebsiella pneumonia* (*K. pneumoniae*) which become one of the major pathogenic bacteria, it responsible for both nosocomial and community-acquired infections because of its high virulence factors and widespread antibiotic resistance. This study looked at the frequency of *K. pneumoniae* in vaginal discharge from patients with vaginitis. It also looked at the molecular relationships between the isolates under investigation and the genes involved in biofilm formation (^{fim}H and ^{mrk}D) and multidrug resistance (^{bla}SHV and ^{bla}TEM) genes. From 1st November 2023 to 30th June 2024, 390 women of reproductive age who visited the gynecology department of Kirkuk General Hospital in northern Iraq for medical attention participated in the study. The data was gathered using a structured questionnaire. After vaginal swabs were collected, they underwent routine microbiological procedures such as culture, Gram stain, biochemical testing, and Vitek-2 Compact System for confirmation purpose. The susceptibility profiles of the *K. pneumoniae* isolates were examined by the Kirby-Bauer disc diffusion technique. Descriptive statistical analyses were also done. Out of 390 vaginal swab samples, 51.79% of samples give positive microbial growth including 5.38% *K. pneumoniae* isolates. Biofilm formation detected among 57.1% of the selected isolates. Regarding Antibiotic susceptibility, all isolates were 100% resistant to Amoxicillin, and Ampicillin. Followed by (85.7%, 81%,76.2%, 71.4%, 66.7%, 61.9%, and 47.6%) resistant to Ciprofloxacin, Cefotaxim, Cefixim, ceftriaxone, Levofloxacin, Azithromycin, Cefepime respectively. There is Low resistant rate (33.3%, and 28.6%) observed against Gentamycin and Amikacin. However, all isolates (100%) were susceptible to imipenem. PCR product reveals high level (100%, and 85.72%) of ^{fim}H, and ^{mrk}D genes respectively. Whereas (66.14%, and 52.8%) of isolates harbor ^{bla}SHV, ^{bla}TEM genes respectively, which explain the occurrence of multidrug resistant *K. pneumoniae* strains among patients with vaginitis. This makes it necessary to implement infection control, good hygiene, and stewardship programs, in order to reduce the prevalence of multidrug resistant strains in healthcare and the community setting.

Keywords: Multidrug Resistant *Klebsiella pneumonia,* Bacterial vaginitis, Biofilm formation, ^{bla}SHV, ^{bla}TEM, ^{fim} H , and ^{mrk}D genes

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1. Introduction

Aerobic vaginitis is characterized by vaginal epithelial inflammation and vaginal dysbioses, which are characterized by a decrease in lactobacilli and an increase in aerobic

and enteric bacteria such as *Escherichia coli*, Klebsiella species, and Enterococcus species [1]. The syndromes of vaginitis include vaginal itching, vaginal discharge, painful urination, and painful sexual relations. Abnormal vaginal discharge in women of reproductive age can result in subfertility, chronic pelvic pain, ectopic pregnancy, early rupture of the membrane, and increases susceptibility to carcinogenic and HIV virus [2]. This bacterium is a Gram-negative opportunistic pathogen that causes infections both in hospitals and in the community. has become a significant public health concern due to the rapid spread of drug-resistant and hyper virulent strains [3,4] It causes numerous common infections, such as urinary tract infections pneumonia, bacteremia, wound infections, and vaginitis [5,6,7].

K. pneumoniae has developed various antibiotic resistance mechanisms, including the acquisition of resistance genes, as to combat commonly used antimicrobials. The *bla*TEM and blaSHV types have been recognized as the most prevalent ESBL genes conferring resistance to beta lactamase and other classes of antibiotic [8]. It was estimated that antibiotic-resistant *K. pneumoniae* accounts for more than 600,000 deaths worldwide several [9]. Another significant aspect of *K. pneumonia* virulence is the formation of biofilms. An aggregate of microorganisms bound to a living or inert surface by a self-produced exo-polymeric matrix comprising extracellular DNA, proteins, and polysaccharides. The bacteria residing in the biofilm are protected from both host immune responses and antibiotic treatments [10] The virulence-associated genes type 1 and type 3 adhesins (*fim*H and mrkD), are crucial for the invasion and attachment of *K. pneumoniae* isolates to host tissues [11,12,13].

Understanding the molecular biological mechanisms behind antimicrobial resistance is crucial for evaluating the effectiveness of intervention strategies [14]. Owing to the significant influence that virulence genes have on the pathogenesis of *K. pneumoniae* isolates and the alarming rise in antibiotic resistance within them, this study looked at the frequency of *K. pneumoniae* in vaginal discharge from patients with vaginitis. It also looked at the molecular level among isolates under investigation the genes involved in biofilm formation (*fim*H and *mrk*D) and beta lactamase resistance (*bla*SHV and *bla*TEM) genes.

2. Materials and Methods

From 1st November 2023 to 30th June 2024, 390 women of reproductive age who visited the gynecology department of Kirkuk General Hospital in northern Iraq for medical attention, had inclusion criteria: should be married, suffered from abnormal vaginal discharge, did not administrated antibiotics for one month, and signed an informed consent participated in the study. The data was gathered using a structured questionnaire. After vaginal swabs were collected by gynecologist, the samples were inoculated directly on the Carry Blair transport media then they underwent routine microbiological procedures such as culture, Gram stain, biochemical testing, according to [15], and used Vitek-2 Compact System for confirmation purpose according to manufacture company (Biomerieux, France).

The binding to Congo red by the studied isolates determined [16]. The Antibiotic susceptibility profiles of the *K. pneumoniae* isolates were examined by the Kirby-Bauer disc diffusion technique and interpretation of resulted inhibition zone was according to the Clinical and Laboratory Standards Institute recommendations [17]. Genomic DNA was isolated from *K. pneumonia* isolates, carried out according to the protocols of DNA extraction kit (Wizard genomic extraction kit, Promega, USA). The extraction protocol for plasmid extraction was applied as directed by the manufacturer (Solarbio Science & Technology Co., Ltd).

The primers used in amplifying the *bla*SHV ,*bla*TEM, *fim*H , and *mrk* D genes products. were designated by the investigators based on standard NCBI gene bank sequence, as listed in the Table 1. These primer were designated via primer design tool at Thermo Fisher Scientific website (https://www.thermofisher.com/en/home.html). In order to reconstitute

the primers, a stock solution was made (Macrogen DNA Technologies, Korea) by adding 250 µl of ddH_2O to each vial of lyophilized primers. This yielded a stock solution of 100 pmol, which was then diluted to 20 pmol/l and kept at (-20 °C). The PCR reactions were performed in a volume of 25µl, consisting of,1µl of forward primer, 1µl of reverse primer, 5µl of DNA template ,12.5µl GoTaq® G2 Green reaction mix (Promega/USA) and up to 25 µl nuclease free water.

The conditions for the PCR amplification were for 4 minutes at 95 ˚C before going through 30 cycles, however, denaturation for 20 seconds at 95 ˚C, then at (59 ˚C, 57 ˚C , 61 ˚C, and 53 ˚C for 30 second) in order to primer annealing for *bla*SHV ,*bla*TEM, *fim*H , and *mrk* D genes respectively, and at the extension was for 50 seconds at 72˚C. The last extension continuously for 10 minutes at 72˚C. Electrophoresis was carried out for separation the PCRs' DNA samples as, they had been run through 1% agarose gel at 80 volt for 45 minutes which stained with 0.5 µl of ethidium bromide (Promega/ USA)). Under UV transilluminator, at 260 nanometer. The resulted DNA bands on the gel were photographed which indicate positive results. Later compared with standard 100bp DNA ladder for detecting their molecular size [18].

Statistical Analysis

SPSS (Statistical Package for Science Services), statistical analysis was performed. Measuring frequencies, and calculating percentages and prevalence were used.

Table 1. Primers and their sequences used in the study

	Gene name	Direction	Sequence of nitrogen bases	GC%	Liquefy degre
1.	*bla*SHV	FORWARD	AGCCGCGGATGACGGGCTGT	65%	64
		REVERSE	GACCGTGCTCGATCACCGCG	65%	65
2.	*bla*TEM	FORWARD	ACTGCGGTGGCACTTGGCTT	55%	62
		REVERSE	GAGTGGCACCTATCTGCGAC	55%	63
3.	*Mrk*D	FORWARD	CTCGTCCTGCGTGGAACCA	55%	66
		REVERSE	GTACTTAGTACCCGGAGGT	45%	62
4.	*fim*H	FORWARD	GAACGCCTTAGGGCTGGTCG	60%	59
		REVERSE	AAGGAGTCGGGCTCAAGTAG	50%	57

3. Results and Discussion

As revealed in figure 1, out of 390 vaginal swab samples, 211 (54.10%) Samples produce positive. microbial growth, including 21(5.38%) isolates of *K. pneumoniae* recovered from cases with vaginitis. This finding is close to other authors [19], where reported (14/214) isolates of *K. pneumoniae* with prevalence of (6.5%). The results of our report is lower than that reported by other researchers in Baghdad [20,21], where they recorded (14.8), and (17.3%) of *K. pneumoniae* of their total samples respectively. Also other investigators reported various prevalence of *K. pneumoniae* strains associated with aerobic vaginitis in other regions, for instance, prevalence of (18.5%) was reported by [22], in Ethiopia, 0.3% [23], and 28.5% [24].

Reproductive tract infections are a constant public health problem throughout the world, it is important to address this threat to ensure females wellbeing [24]. Aerobic vaginitis; is an inflammation of vaginal epithelial cells, in addition to dysbioses Characterized by reduction in normal vaginal lactobacilli and increase abnormal vaginal microflora including aerobic and facultative an aerobic enteric bacteria as klebsiella species, *E. coli*, Staphylococcus species [25], *K. pneumoniae* consider one of the most pathogenic microbe

causing. Health care associated infection due to the high virulence factors and multidrug resistant *K. pneumoniae* [1].

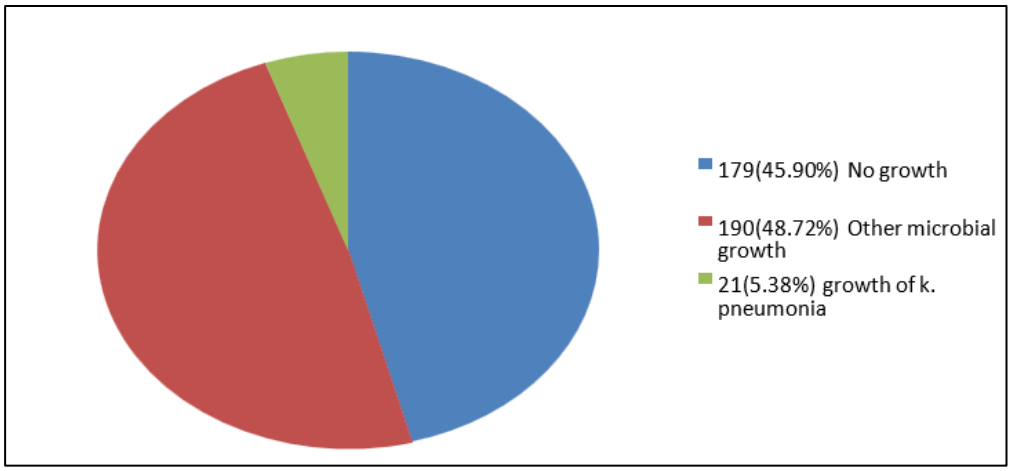


Figure 1. Percentage of *k. pneumonia* and other microbial growth from vaginal discharge samples

The cultured *k. pneumonia* strains grew as mucoid pink colony on MacConkey agar. as well as had the ability to grow on Congo red medium and (57.1%) of the studied isolates produce positive reaction. They found to have a high invasive capacity and epithelial cells cytotoxicity with biofilm formation. This result lower than others [26], who reported (91.7%) biofilm formation among *K. pneumonia* isolates from clinical samples. Moreover, other researchers evaluated biofilm formation using microliter plate method and demonstrates that all fifty-six *K. pneumonia* strain produced biofilm. [5], whereas Karimi, Zarei [27], reported (75%) of their *K. pneumonia* isolates of clinical samples had the ability to biofilm formation.

The variation in biofilm formation among *K. pneumoniae* strain in our results and previous reports might be attributed to variation in the studied conditions, methods, locations of study and the level of genetic expression [5]. The ability of enteric bacteria to produce biofilm [28,29,5] aid them in escaping immune system responses of the host and confer resistance to antibiotics [30].

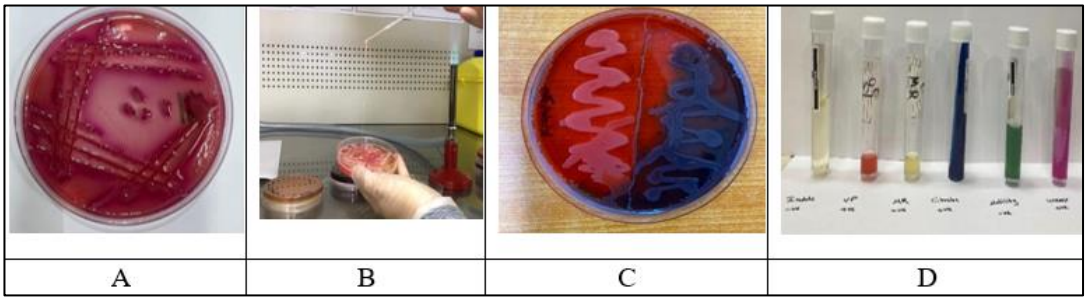


Figure 2. Cultural and biochemical characteristics of *K. pneumonia* isolates; A/ Growth of pink *K. pneumonia* colonies on MacConkey agar; B/ Mucoid colony of *K. pneumonia* on MacConkey agar; C/ Growth of *K. pneumonia* colonies on Gongo red agar; Growth produce blue pigmentation at left side indicate positive reaction and biofilm formation; D/ Biochemical reaction of *K. pneumonia*

As shown in Table 2; all 21(100%) isolates of *K. pneumoniae* were resistant to amoxicillin and ampicillin. Also A high rate of resistance was observed for Ciprofloxacin (85.7), Cefotaxim (81%), cefixim (16.2%) and Ceftriaxon (71.4%). Whereas, there were relative

moderate resistance expressed against Levofloxacin (66.7%), Azithromycin (61.9%). and Cefepime (47.6%).

While low resistance rate was demonstrated against Amikacin (28.6%) and Gentamycin (33.3%). However, all isolates were sensitive to Impenem (100%). This result in agreement with other investigators who reported the resistance of *K. pneumoniae* isolates to multiple beta lactam antibiotic classes with a high sensitivity to Amikacin and Carbapenem antibiotics [31,32]. The current study demonstrates multidrug drug resistant *K. pneumoniae* srains which resist to at least three classes of antibiotics. Hence the treatment of bacterial vaginitis caused by these strains become difficult with applied antibiotics in hospitals. The shift in normal vaginal flora from Lactobacillus species to opportunistic pathogenic bacteria like *K. pneumoniae* with their ability to confer resistant to varies antibiotic agents is dangerous, reduce treatment options for patients and delay effective treatment [22].

Many researchers were reported the ability of *K. pneumoniae* strains to produce different types of beta-lactamase enzymes which confer resistance to various types of beta-lactam and other classes of antibiotics [33,31]. Accordingly, accurate diagnosis of the genital tract infections should precede the treatment of bacterial vaginitis with appropriate antibiotic therapy [20].

Table 2. Antibiotic susceptibility of *K. pneumoniae* isolates

K. pneumonia / Antibiotics	Resistant %	Sensitive%
Amoxicillin	100.0	0.0
Ampicillin	100.0	0.0
Azithromycin	61.9	38.1
Ciprofloxacin	85.7	14.3
Levofloxacin	66.7	33.3
Cefixime	76.2	23.8
Cefotaxime	81.0	19.1
Ceftriaxone	71.4	28.6
Cefepime	47.6	52.4
Amikacin	28.6	71.4
Gentamycin	33.3	66.7
Imipenem	0.0	100.0

Four virulence isolated bacteria had been detected in studied isolates, including beta-lactamase genes, ^{bla}SHV and ^{bla}TEM which represent 14 (66.67%), and11(52.38%) of the studied isolates respectively. While ^{fim}H gene detected in all strains 21(100%) which encoding biofilm formation. Moreover, ^{Mrk}D gene 18 (85.72%) which related to bacterial fimbriae, adhering capacity to the host cells and biofilm formation.

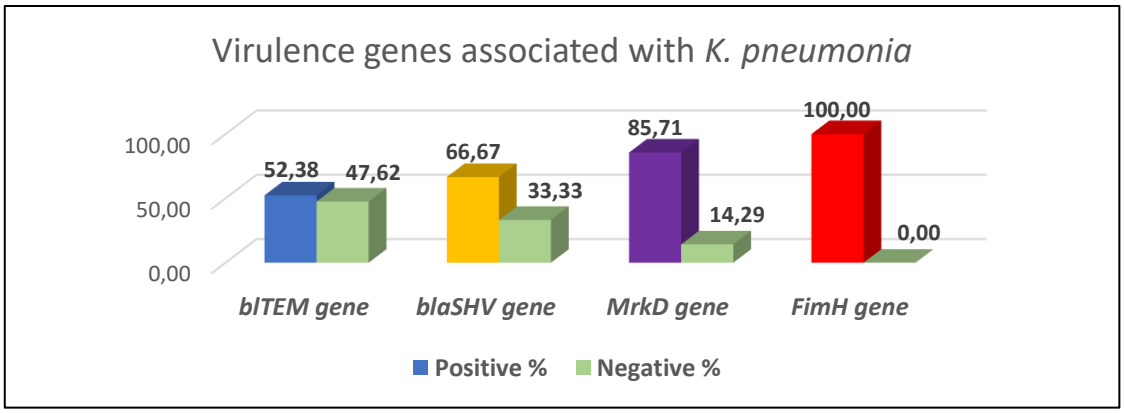
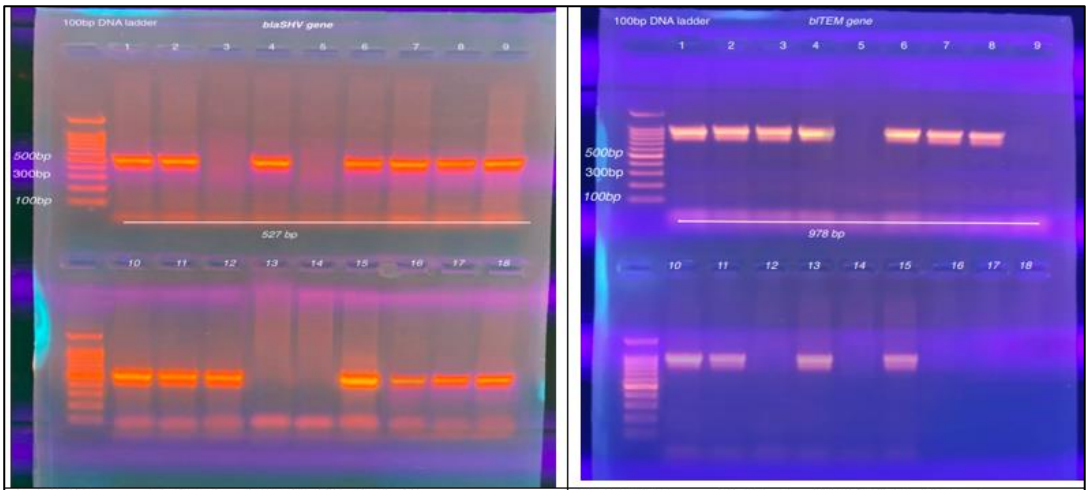


Figure 3. Demonstrates the percentages of virulence factors genes exhibit by *K. pneumoniae*. isolates from vaginal discharge Samples

The polymerase chain reaction reveals distribution of ^{bla} SHV and ^{bla}TEM genes at considerable level which explain the multidrug resistant to beta-lactam and some other classes of antibiotics investigated in this study. This finding in line with other authors who reported various level of ^{bla}SHV and ^{bla}TEM in their local bacterial strains [31]. Ghenea *et al.* [33], reported presence of ^{bla}SHV in all 32 studied *K. pneumoniae* strains. Whereas, ^{bla}TEM observed in only 14 strains. There are more than 100 different SHV and 140 TEM types beta-lactamases genes are encountered in bacterial strain belong to the family Enterobactericeae including *K. pneumoniae* [34,33].

Currently, the most used therapeutic strategy for treating *K. pneumoniae* infections is the beta-lactam antibiotics [35]. However, the random and over use of these antibiotics accelerate evolution of antibiotic resistant genes of the bacterial cells [36] This will reduce the chance of treating infected patients and elevate the rate of morbidity and mortality [37]. Consequently, it is imperative to comprehend the molecular mechanisms underlying antibacterial agent resistance in order to create innovative intervention strategies that will increase patient survival [38,39,40,41].



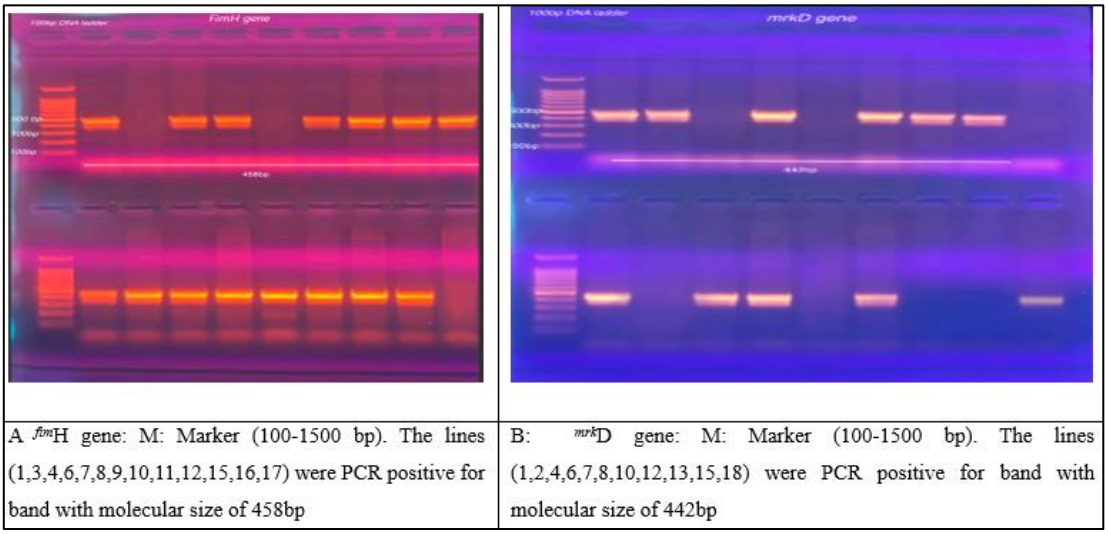
A: ^{bla}SHV gene: M: Marker (100-1500 bp). The lines (1,2,4,6,7,8,9,10,11,12,15,16,17,18) were PCR positive for band with molecular size of 527bp

B: ^{bla}TEM gene: M: Marker (100-1500 bp). The lines (1,2,3,4,6,7,8, 10,11,13, 15) were PCR positive for band with molecular size of 978bp

Figure 4. DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotide primer was used for detection of A: blaSHV and B: blaTEM gene in K. pneumonia isolates

In regard to the results of molecular investigation in the current study by using PCR technique, the biofilm formation genes; (^{fim}H, and ^{mrk}D) have been detected in majority of

studied *K. peumoniae* strains. The finding of current study is similar to that reported by [42], who revealed 100% of isolates harbour *Mrk*D. However another previous study reported lower level (87.5%) and (53.6%) of *fim*H, and *Mrk*D genes respectively among 56 *K. pneumoniae* isolates [5]. This variation in prevalence of biofilm formation and antibiotic resistance genes might be contributed to differences in studied conditions, geographic variation in distribution of such genes in microorganisms.



A *fim*H gene: M: Marker (100-1500 bp). The lines (1,3,4,6,7,8,9,10,11,12,15,16,17) were PCR positive for band with molecular size of 458bp	B: *mrk*D gene: M: Marker (100-1500 bp). The lines (1,2,4,6,7,8,10,12,13,15,18) were PCR positive for band with molecular size of 442bp

Figure 5. DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotide primer was used for detection of A: *fim*H and B: *mrk* D genes in *K. pneumonia* isolates

4. Conclusion

It have been concluded that majority of isolated *K. pneumoniae* from vaginal secretion circulating in the Kirkuk General Hospital, Iraq, were multidrug resistant strain and harboring high level of virulence factor genes *bla*SHV, *bla*TEM, *fim* H, and *Mrk*D which are participate in conferring. resistance to current used antibiotics, and biofilm formation which had a crucial role in pathogenicity associated with *K. pneumoniae* infections. This makes it necessary to implement infection control, good hygiene, and stewardship programs, in order to reduce the prevalence of multidrug resistant strains in healthcare and the community setting.

Ethical Clearance

All experimental protocols were approved under the College of Education for pure science/ Biology department/University of Kirkuk. Iraq and all experiments were carried out in accordance with approved guidelines.

Conflict of interests

The authors declare they have no conflict of interest.

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