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Molecular Characterization of *Proteus mirabilis* Recovered From Milk and Dairy Products

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Abstract

GLOBALLY, *Proteus* spp. is widespread in many foods and animals, presenting a significant health obstacle. The present investigation found that *Proteus mirabilis* (*P. mirabilis*) was recorded in 19 (12.67%) samples of 150 examined dairy products. The occurrence rates of *P. mirabilis* in the analyzed samples of raw milk, Kariesh cheese, Tallaga cheese, Feta cheese, yoghurt, and ice cream were 20%, 28%, 16%, 8%, 4%, and 0%, respectively. The antimicrobial resistance of the isolated *P. mirabilis* strain was assessed, revealing their resistance to several antibiotics. All *P. mirabilis* isolates exhibited full resistance to erythromycin, whereas 94.7% were resistant to tetracycline and penicillin-G. The detection of the marker gene, the *UreR* gene, by using PCR confirmed the detection of *P. mirabilis*. The presence of this gene was observed in all 19 *P. mirabilis* isolates. The genetic analysis of *Proteus* spp. was conducted to identify the presence of *LuxA, FlaA*, *mrpA*, and *hpmA* genes, which are known to be associated with virulence in P. mirabilis. Out of the 19 *P. mirabilis* isolates, the presence of the *luxA* gene was identified in 12 of them. The presence of the *FlaA* gene was observed in all *P. mirabilis* isolates. In conclusion, Kariesh cheese had significantly the highest prevalence rate, followed by raw milk. Therefore, strict hygienic measures should be followed during raw milk collection, and the manufacture of dairy products to avoid contamination of such products with enteric bacteria such as *P. mirabilis*.

Keywords: *P. mirabilis*, dairy products; drug resistance; virulence genes.

Introduction

The genus *Proteus* is a Gram-negative and rod-shaped bacterium from the *Enterobacteriaceae* family. This family is categorized in the tribe Proteeae, which includes the genera Morganella and Providencia [1]. Members of the genus *Proteus* are widely distributed in both natural environments and the gastrointestinal tracts of people and animals [2]. *Proteus* spp. is regarded as indicators of fecal contamination [3]. *Proteus* spp. poses a considerable challenge to both humans and animals worldwide, being prevalent in both food and animal species. Multiple strains of this bacterium have already exhibited resistance, indicating a considerable challenge to food safety [4]. Numerous cases of food poisoning have been attributed to *Proteus* spp., alongside an increasing prevalence of foodborne diseases caused by this pathogen. Consequently, it is essential to establish control programs and preventive measures to efficiently halt and manage outbreaks of foodborne illnesses and poisoning [5]. The transmission of antimicrobial-resistant bacteria from food to humans can occur via cross-contamination [6]. *Proteus* spp. generally disseminates via the fecal-oral route, entailing the transmission of bacteria from feces to hands or surfaces [7]. *Proteus* spp. is recognized for causing complicated urinary tract infections (UTIs) more often than other uropathogens. It may also lead to the formation of urinary calculi. Moreover, *Proteus* spp. can induce infections in the respiratory system, ear, nose, skin, and wounds, as well as meningitis in neonates or babies. It has also been linked to rheumatoid arthritis and wound infections. *Proteus*

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spp. can, in certain instances, induce gastroenteritis [8]. *Proteus mirabilis* accounts for 90% of *Proteus* spp. infections and is primarily found in persons with compromised immune systems [9]. *P. mirabilis* is found in soil, sewage, water, and fecal matter and is considered a frequent microbe in humans and animals' gastrointestinal tract (GIT) [10]. According to Alatrash and Al-Yaseen [11], people who get urinary tract infections from *P. mirabilis* often have cystitis, bacteriuria, renal and bladder calculi, catheter obstruction due to stones, and severe pyelonephritis. *Proteus* spp., and other members of the enteric bacteria were encountered in several health issues in various animal species [12, 13, 14].

Antimicrobial resistance constitutes a significant global challenge. Bacteria develop antibiotic resistance via multiple processes, including the overuse of antimicrobials in livestock production and animal farming. These resistant strains may subsequently be transmitted to humans via the ingestion of contaminated animal-derived food [15, 16].

These bacteria's pathogenicity is linked to their multiple virulence factors, including pili (fimbriae), flagella, urease, protease, and hemolysin, among others. Numerous pathogenic factors in Proteus spp. are regulated by virulence genes expressed inside operons [17, 18]. The *ureC1* gene, encoding urease, is essential for the pathogenicity of *P. mirabilis*. This enzyme promotes the production of kidney and bladder stones or impedes the flow of indwelling urinary catheters [19]. The formation of urolithiasis necessitates urease to decompose urea into ammonia. This mechanism raises urine pH, leading to the synthesis of calcium and magnesium compounds and the subsequent development of urinary calculi [19]. The pH alteration is critical for *P. mirabilis* colonization of catheters. It facilitates bacterial adhesion and biofilm formation [20, 21].

P. mirabilis has been found to contain a variety of fimbriae. The primary variety is *MR/P* fimbria, encoded by the genes *mrpA, mrpB, mrpC, mrpD, mrpE, mrpF, mrpG,* and *mrpI*. The *mrpA* gene is important for bacteria to be harmful because it helps them stick to epithelial tissue, make biofilms, and swarm [22]. The mannose-resistant/proteus-like fimbriae (*MR/P*) are linked to infections in the urinary tract that affect the bladder and kidneys [23]. The *mr/p* gene cluster has two transcripts: *mrpABCDEFGHJ* (Operon) and *mrpI*. The main structural part is the *mrpA* protein, which is needed for the first stage of infection, which includes cluster formation, and for maintaining normal levels of bladder colonization in later stages [24]. Fimbriae facilitate cell adhesion to uroepithelial cells, aid in motility against urinary flow, enable nutritional absorption, and combat the host's immunological response. *P. mirabilis* contains 17 distinct genes that encode various fibril structures. *MR/P* (mannose-resistant Proteus-like pili), *PMP* (*P. mirabilis* P-like pili), *PMF* (*P. mirabilis* fimbriae), and *UCA* (uroepithelial cell adhesin) are the most important ones [1, 25]. A separate group of genes linked to virulence comprises the quorum sensing genes (*luxS* and *rsbA*). The *luxS* gene emits a signal that indicates interactions among different species and cell density within a polymicrobial community. This is crucial for regulating genes that enhance viral potency [26].

The *rsbA* gene encodes a histidine-rich phosphotransmitter within the bacterial 2-component signaling system. This gene regulates the movement pattern referred to as swarming. It encodes a protein that functions as a sensor for environmental variables [26]. Besides, Abbas et al. [20] indicated that *rsbA* facilitated the synthesis of biofilm and extracellular polysaccharides. The hemolytic activity of *P. mirabilis* is linked to the proteins *hpmA* and *hpmB*, which function as hemolysins. The primary function of *hpmA* is to induce tissue damage. The activation of *hpmA* occurs when its N-terminal peptide is cleaved [19]. The authors stated that *hpmA* activation and trafficking are dependent on *hpmB* hemolysin. Hemolysin is believed to be important in urinary tract infections caused by *P. mirabilis* because it increases the bacteria's propensity to incite infections [27]. *Proteus spp.* possesses the capability to encode and synthesize *hlyA*, a variant of hemolysin protein. The virulence genes in *P. mirabilis*, such as urease, hemolysin, fimbriae, and flagella, help it stay alive in the urinary tract [28].

The research reveals a significant gap on the presence of *P. mirabilis* in dairy products, specifically for its antibiogram and molecular features. In a previous study, *P. mirabilis* was recovered from raw milk and bovine mastitis in Egypt [29]. However, continuous monitoring for the prevalence of *P. mirabilis* in milk and other dairy products retailed in Egypt is highly recommended. This study was conducted to investigate the prevalence of *P. mirabilis* in milk and dairy products available in Egypt. The recovered isolates were additionally examined for antimicrobial resistance profiling. PCR was utilized to identify virulence-associated genes.

Material and Methods

Collection of Samples

A total of 150 random samples of milk and its products, including raw milk, Kariesh cheese, Tallaga cheese, Feta cheese, yoghurt, and ice cream (25 of each), were gathered from various markets and retail stores in Benha city, Kalyobia government, at varied time intervals. Each sample was carefully stored in a separate plastic bag and promptly transported to the laboratory in an insulated ice box, ensuring complete sterility without unnecessary delay. The collected samples were promptly tested to

detect any contamination with *Proteus mirabilis* and assess their hygienic condition.

Ethical considerations:

The study obtained ethical permission number BUFVTM03-02-06-24 from the Research Ethics Board at Benha University's Faculty of Veterinary Medicine to use animal and human samples on a nationwide scale. It notes worthy to mention that all samples were purchased from the market and no pain was introduced to any animals or human.

Proteus species detection:

Sample preparation:

Under strictly sterile circumstances, 25 ml of milk (equivalent to 25 g of milk products) were measured and put into a sterile flask designed for homogenization. The flask included 225 ml of sterile peptone water (0.1%). The resulting mixture was then adjusted to an alkaline pH of 8 [30].

Proteus species isolation:

Each previously produced tube was inoculated into Violet Red Bile Glucose agar (VRBG) (Oxoid, UK) plates and thereafter incubated at 37°C for 24 hours. The resultant growth was subsequently sub-cultured on MacConkey agar and incubated at 37°C for an additional 24 hours. The putative typical colonies (purplish red colonies) were purified and subsequently placed into tilted nutrient agar tubes for additional identification. The identification of pale colonies, indicative of non-lactose fermenting bacteria, was conducted by culturing them on Xylose-lysin-deoxycholate (XLD) agar and blood agar (Oxoid, UK). This was executed to identify swarming. The suspected isolates of *Proteus* spp. were identified based on the methodology described by MacFaddin [31]. The recovered isolates were subjected to morphological examination, motility test, and different biochemical tests. These tests included indole, methyl red, Voges Proskauer, citrate utilization, urease, hydrogen sulphide production, gelatin liquefaction, nitrate reduction, Ornithine decarboxylase detection, L-lysine decarboxylase detection, Arginine decarboxylase detection, sugar fermentation, oxidase activity, and catalase activity.

Antimicrobial susceptibility testing of isolates (Antibiogram):

Following the protocol outlined by the Clinical and Laboratory Standards Institute "CLSI" [32], the antimicrobial susceptibility of the isolated *Proteus species* was evaluated using the single diffusion method. We used sensitivity discs from Oxoid Limited (Basingstoke, Hampshire, UK) with different concentrations for antimicrobial susceptibility testing. The agar plate method was employed to ascertain antibiotic sensitivity; the tested bacterium was cultured on Mueller–Hinton agar (Oxoid, UK). The Muller Hinton agar surface was uniformly covered with the bacterial culture. The result was the infected plate being placed on top of the antibiotic discs. In addition, after that, the plate was incubated in an incubator set at 25 °C for 2–7 days. Next, the plate was checked for the presence of any bacteria around the antibiotic discs. In terms of effect on microbial growth, the antibiotic with the wider inhibition zone is thought to be the most potent. We followed the guidelines provided CLSI [32], when testing for antibiotic susceptibility was carried out. Each of the studied strains was ranked according to its susceptibility, intermediate status, or resistance.

Identification of P. mirabilis using PCR [33]:

A biomarker for the validation of *P. mirabilis* detection was identified by identifying the *UreR* gene. Additionally, the primers listed in Table 1 were used to demonstrate the presence of *P. mirabilis* virulence genes, which include the following: biofilm formation gene (*LuxA*), flagellar gene (*FlaA*), fimbria formation gene (*mrpA*), and hemolysin gene (*hpmA*). Here, 1 mL of broth that had been incubated overnight was spun at 13,000 rpm for 2 minutes at 4 °C to achieve the desired result. DNA extraction was performed according to the instruction manual of kits (Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721). The 25 µL total volume was used to run the PCR reaction consisted of 5 µl of the bacterial lysate, 5 µl of 10x assay buffer for Taq polymerase containing 1.5 mM MgCl2, 2 µl of 10mM dNTP mix 1 µl each of forward and reverse primer (10 pmol) and 1.25 U of Taq DNA polymerase made upto 25 µl using sterile distilled water. The PCR run for these genes was done according to the following protocol: 30 cycles, starting with a denaturation step at 94°C for 5 minutes. One minute of denaturation at 94°C, followed by 30 seconds of annealing at 58°C, one minute of extension at 72°C, and five minutes of final extension at 72°C made up each cycle. After that, a 1.5% agarose gel was electrophoresed on a voltage of 80 V for 90 minutes with the amplified samples. After the samples were treated with ethidium bromide, they were examined under ultraviolet light. To determine the molecular size, a DNA ladder consisting of 100 base pairs was used. Using both positive and negative controls, all of the tested isolates were examined for the genes that were the subject of the study.

Statistical analysis:

Statistical Package for the Social Sciences, version 25 (IBM Corporation, Armonk, NY, USA), was used to encode, enter, arrange, and analyze the gathered data. A collection of categories was used to convey the qualitative data, together with the percentage, frequency, or proportion of each category. The $p<0.05$ level of significance was utilized.

Results and Discussion

In the current study, the overall prevalence of *Proteus mirabilis* in examined milk and dairy products was 12.67% (19 out of 150 samples). The prevalence rates of *P. mirabilis* were 20%, 28%, 16%, 8%, 4%, and 0% in the examined samples of raw milk, Kariesh cheese, Tallaga cheese, Feta cheese, yoghurt, and ice cream (Fig. 1). The high prevalence in the Kareish cheese could be attributed to selling of that kind of cheese open to air in the market and the method of preparation. This result is regarded as higher than Syed [34] who found that 18% of milk and milk products (curd and ice cream) samples were contaminated with Proteus, and Sobeih et al. [35], isolated *Proteus vulgaris* from 13.10% from raw milk samples, and 4.76 % from ice cream samples. While lower isolation rates was recorded by Awad et al. [36] who showed that *Proteus* spp. was not detected in the examined raw milk samples but could be isolated from Damietta cheese samples at 8%, and from Kareish cheese at 4%. Such variations in the prevalence rates could be attributed to the differences in the hygienic condition of the dairy processing plants and difference in the contamination load of the dairy products.

The antimicrobial resistance of the recovered *P. mirabilis* isolates was tested and the recovered *P. mirabilis* isolates showed resistance to more than one antibiotic. As 100% of *Proteus mirabilis* isolates showed complete resistance to Erythromycin, and 94.7 % of *Proteus mirabilis* isolates were resistant to tetracycline and Penicillin-G. The lowest degree of resistance was detected towards Meropenem at 5.3%. While, *Proteus mirabilis* isolates were susceptible to Cefepime and Ipipenem at 84.2% (Fig. 2). This result agrees with the result obtained by Al-Oqaili et al. [37] and AL-Ta'ee [38] who reported that most isolates of *Proteus* spp. are considered to be resistant to Tetracycline in rate reach to 96.8% and 96%, respectively. Contrarily, a 72.1% sensitivity rate to tetracycline was discovered by Akerele et al. [39]. Consistent with current research results, Dadheech et al. [40] found that all Proteus species in the Ajmer Region of India were resistant to tetracycline, this investigation found that 94.7% of *P. mirabilis* were resistant. According to Aliyu et al. [41], tetracycline is commonly used for chemotherapy and routine prophylaxis in Nigerian cattle management. Our results are consistent with previous research showing that antibiotic resistance has grown among *P. mirabilis* isolates; however, Fallah et al. [42] in Iran found a greater rate of resistance to ciprofloxacin, ceftriaxone, and imipenem.

Agarose gel electrophoresis of PCR of the *UreR* gene (225 bp) was employed for confirmation of *P. mirabilis*. This gene was detected in all *P. mirabilis* isolates (19 strains) (Fig. 3).

Proteus spp. possess the ability to produce urease and raise the pH of urine by breaking down urea into NH4, enabling them to thrive in a favorable environment. This leads to the accumulation of organic and inorganic substances, which ultimately leads to the formation of struvite stones. Struvite stones consist of struvite, which is a compound of magnesium ammonium phosphate, and apatite, which is a compound of calcium carbonate. Proteus mirabilis is responsible for the development of urinary stones and the buildup of encrustations on indwelling catheters, which worsens the persistence of the infection. The presence of stones around the bacterium makes antibiotic treatment ineffective. Urease facilitates the breakdown of urea into CO2 and NH4 through hydrolysis. This process raises the pH of the surrounding environment and prevents the formation of naturally soluble polyvalent ions from urine, particularly magnesium, ammonium, phosphate, and calcium ions. As a result, struvite and carbonate hydroxyapatite crystals, which are the main components of urinary stones, are formed [28]. The presence of the urease gene cluster in *P. mirabilis* is extensively established, comprising the genes *ureABC* and *ureDEFG*. The *ureABC* genes encode the structural subunits of the apoenzyme, while the *ureDEFG* genes are responsible for producing the proteins that allow the insertion of the necessary nickel ions into the catalytic site [43]. *UreR* is the causative factor for initiating the expression of the urease operon. *UreR*, a transcriptional regulator belonging to the *AraC/XylS* family, enhances the expression of the ure gene cluster when urea is present [44, 45]. All the obtained isolates of *P. mirabilis* in the present investigation were found to contain the *ureR* gene. In contrast to Ridha Abbas Al-Fahham and Raoof Kareem [46], who observed the presence of the *ureR* gene in 14 out of 15 *P. mirabilis* isolates, indicating a frequency of 93.3%.

A genetic investigation was conducted on *Proteus species* to identify the presence of *LuxA, FlaA, mrpA,* and *hpmA* genes, which are known to be associated with virulence in *P. mirabilis*. Out of the 19 *P. mirabilis* isolates, the presence of the *luxA* gene was observed in 12 isolates. The *FlaA* gene was identified in every *P. mirabilis* isolate. The *mrpA* gene was identified in six *P. mirabilis* isolates. The *hpmA* gene was identified in 11 *P. mirabilis* isolates (Fig. 4).

The current investigation revealed lower prevalence rates of the target virulence genes compared to the findings of Ali and Yousif [47], who reported greater prevalence rates of the *flaA* (86.66%) and *hpmA* (100%) genes. The presence of virulence factors, such as *hpmA* hemolysin and MR/P fimbriae, is observed in most strains, irrespective of their source of isolation (95, 96). For instance, the presence of *hpmA* was detected in all 63 isolates examined by Southern blot analysis, and this was

found to be associated with hemolytic activity [48]. Similarly, a study involving 211 isolates utilized a combination of PCR and dot blot techniques, and it was observed that all of them included the *hpmA* gene [27]. Flagella are subject to attack by the innate immune system and are also costly to manufacture in terms of energy expenditure [49]. Similarly, while the majority of flagellar genes are preserved in *P. mirabilis*, the gene responsible for the whip structure exhibits significant variability, as observed in other bacterial species. *P. mirabilis* possesses an additional flagellar gene called *flaB*, which remains inactive. However, it has the ability to undergo recombination with *flaA*, resulting in the production of hybrid *FlaAB* flagella [50]. In this study, the prevalence rates of the target virulence genes were lower compared to the findings of Ali and Yousif [48]. They reported higher prevalence rates of the *ureC* (100%), *ure*A (96.66%), *flaA* (86.66%), *hpmA* (100%), and *zapA* (100%) genes in the recovered *P. mirabilis* in their study. In general, detection of such virulence factors in the recovered isolates throw the light on the possibility of causing infections and gastrointestinal diseases to the consumers.

Future studies are still needed to investigate the seasonal effect on the prevalence of *Proteus* spp. in different dairy products and to include other locations such as urban and rural sites and to study their effect on the contamination rate of dairy products with *Proteus spp*.

Conclusion

This study demonstrated isolation of *P. mirabilis* from retailed raw milk and dairy products at variable rates. The recovered isolates showed marked drug resistance and harbored several virulence-associated genes. Therefore, strict hygienic measures should be followed during collection of raw milk at retail and during the manufacture of dairy products to avoid contamination of such products with enteric bacteria. In addition, continuous monitoring programs are highly recommended to involve other locations in Egypt to investigate the prevalence of multidrug resistant Proteus spp. Besides, legislations to inhibit selling Kariesh cheese and other dairy products open to air in the markets are highly suggested.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' contributions

All authors contributed equally to this study.

TABLE 1. The primers used in the current study

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
UreR (F)	GGTGAGATTTGTATTAATGG	225	**Zhang et al. [51]**
UreR (R)	ATAATCTGGAAGATGACGAG		
LuxA (F)	GGCACCAGATTCAACTTTCAAG	290	**Al-Oqaili et al. [52]**
LuxA (R)	GACCCCAAGTTTCCTGTAAGTG		
FlaA (F)	ATCAATGCAGCTGCGACACT	445	
FlaA (R)	TGAAGTACCCGCTTGTTGCA		
mrpA (F)	ATGAAATTAAATAAATTAGC	525	**Mirzaei et al. [53]**
mrpA (R)	CTG ATAAGTCAGTGCGAAAG		
hpmA (F)	ATAGTCACGCCAAATAACGAA	971	
hpmA (R)	TATTTCCACGAGTAGAACCAG		

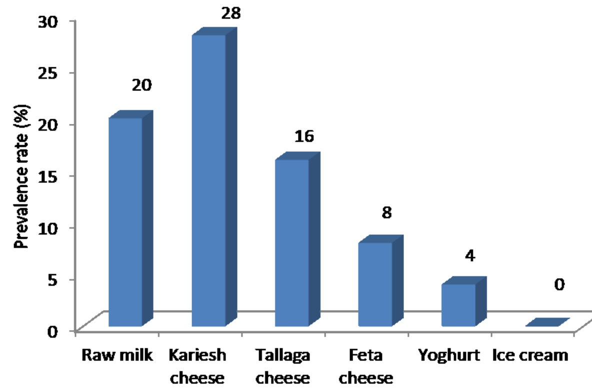

Fig. 1. Prevalence rate (%) of *P. mirabilis* in the examined milk and dairy product samples (n= 25/ each product)

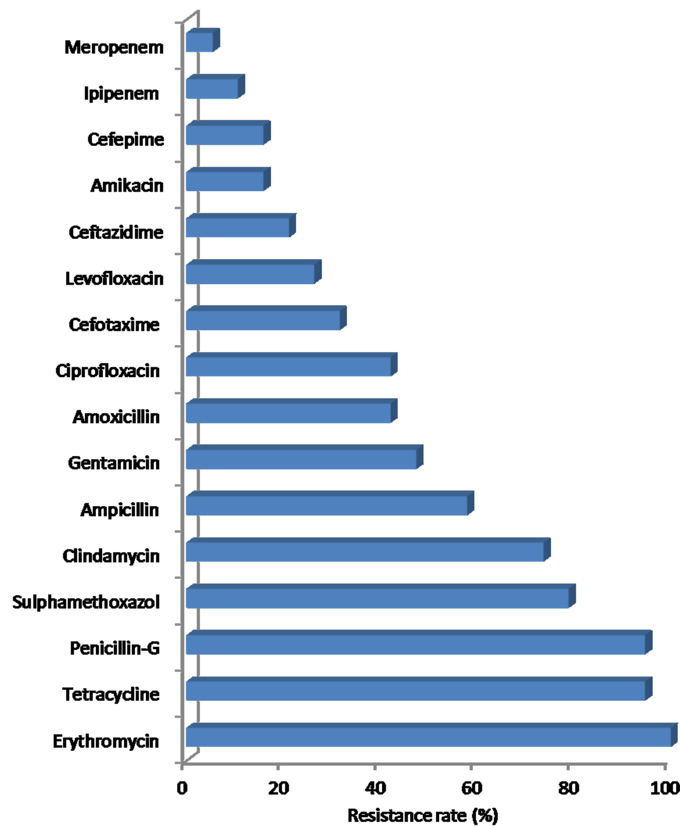

Fig. 2. Antimicrobial resistance rate (%) of the identified *P. mirabilis* isolates (n = 19 isolates)

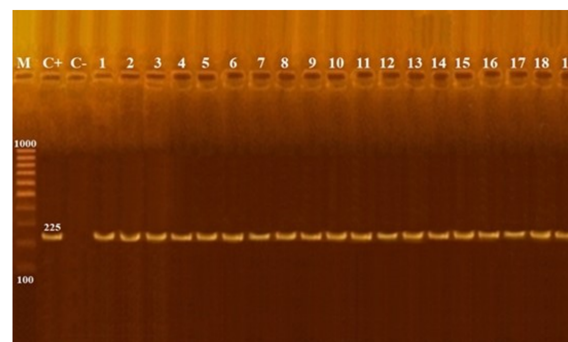

Fig. 3. Agarose gel electrophoresis of PCR of *UreR* gene (225 bp) for confirmation of *P. mirabilis*.
Lane M: 100 bp ladder as molecular size DNA marker. **Lane C+:** Control positive
Lane C-: Control negative **Lane 1:** Control positive *P. mirabilis* for *UreR* gene.
Lane 12: Control negative. **Lanes from 1 to 19:** Positive strains for *UreR* gene.

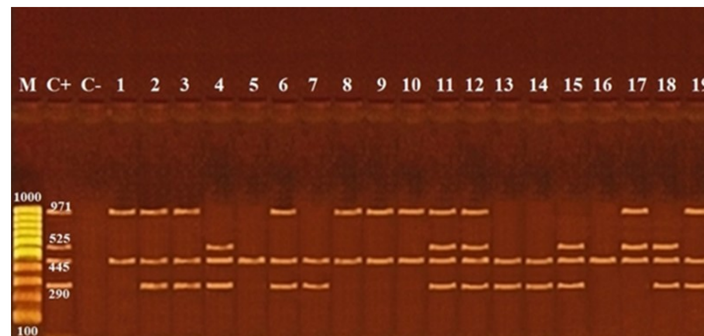

Fig. 4. Agarose gel electrophoresis of multiplex PCR of *LuxA* (290 bp), *FlaA* (445 bp), *mrpA* (525 bp) and *hpmA* (971 bp) as virulence genes of *P. mirabilis.*

Lane M: 100 bp ladder as molecular size DNA marker.
Lane C+: Control positive **Lane C-:** Control negative
Lane 1: Control positive for *LuxA, FlaA, mrpA* and *hpmA* genes.
Lanes 11 &12: Positive strains for *LuxA, FlaA, mrpA* and *hpmA* genes.
Lanes 2, 3, 6 &19: Positive strains for *LuxA, FlaA* and *hpmA* genes.
Lanes 4, 15 &18: Positive strains for *LuxA, FlaA* and *mrpA* genes.
Lane 17: Positive strain for *FlaA, mrpA* and *hpmA* genes.
Lanes 1, 8, 9 &10: Positive strains for *mrpA* and *hpmA* genes.
Lanes 7, 13 &14: Positive strains for *LuxA* and *FlaA* genes.
Lanes 7: Positive strain for *FlaA* gene.

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التوصيف الجزيئي لبكتيريا بروتيوس ميرابيليس المعزولة من الحليب ومنتجات الألبان

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الملخص

عالمياً، تنتشر أنواع البروتيوس في العديد من الأطعمة والأغذية، مما يشكل خطورة علي الصحة العامة. أوضح الدراسة الحالية عزل بكتيريا بروتيوس ميرابيلس من 19 (12.67%) عينة من أصل 150 منتج ألبان تم فحصها. كانت معدلات عزل هذه البكتريا من منتجات الالبان محل الدراسة 20% من الحليب الخام،28 % من الجبن القريش، 16% من جبن الثلاجة، 8% من جبن فيتا ، 4% من الزبادي، و0% من الآيس كريم، على التوالي. كما تم تقييم مقاومة المعزولات للمضادات الميكروبية، مما كشف عن مقاومتها لعدة مضادات حيوية. أظهرت جميع عزلات بروتيوس ميرابيلس مقاومة كاملة للإريثروميسين، في حين كانت 94.7% مقاومة للتتراسيكلين والبنسلين-ج. تم إجراء التحليل الجيني لأنواع بروتيوس ميرابيلس لتحديد وجود جينات LuxA و FlaA و mrpAوhpmA، والتي تُعرف بأنها مرتبطة بالضراوة ، تم تحديد وجود جين luxA في 12 من المعزولات. تمت ملاحظة وجود جين FlaA في جميع عزلات بروتيوس ميرابيلس يستخلص من هذه الدراسة أن أعل معدل تلوث للعينات سجل في الجبن القريش ، يليها الحليب الخام. لذلك، يجب اتباع إجراءات صحية صارمة أثناء تجميع الحليب الخام وتصنيع منتجات الألبان لتجنب التلوث بالبكتيريا المعوية مثل بروتيوس ميرابيلس.

الكلمات الدالة: بروتيوس ميرابيلس ، منتجات الألبان، مقاومة الأدوية، جينات الضراوة.