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Isolation and antimicrobial susceptibility of *Proteus vulgaris* isolated from milk and dairy products

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ABSTRACT

The isolation of *Proteus spp*., particularly *Proteus vulgaris (P. vulgaris)* from food subjects indicates fecal contamination. In addition, the uncontrolled usage of antimicrobials during livestock production led to drug resistance among foodborne pathogens. Therefore, this study aimed to investigate the prevalence of *P. vulgaris* from raw buffalo milk and dairy products, including Kariesh cheese, low salt (Tallaga cheese), Feta cheese, yoghurt, and ice cream, randomly collected from Benha city, Egypt. In addition, the antimicrobial susceptibility testing of the recovered *P. vulgaris* isolates was screened. The present investigation found that the total prevalence of *P. vulgaris* in the analyzed raw buffalo and dairy products was 6%, with 9 out of 150 samples testing positive for the bacteria. The occurrence rates of *P. vulgaris* were 12%, 16%, 4%, 0%, 4%, and 0% in the samples analyzed in un-pasteurized (buffalo milk), Kariesh cheese, Tallaga cheese, Feta cheese, yogurt, and ice cream. *Proteus vulgaris* isolates exhibited multi-drug resistance. All *P. vulgaris* isolates showed full resistance to erythromycin and clindamycin, whereas 77.8% of the isolates showed resistance to sulphamethoxazol. The resistance to ceftazidime, meropenem, and imipenem was found to be at the lowest degree, with a detection rate of 11.1% for each. Therefore, strict hygienic measures should be followed during the manufacture of dairy products to avoid their contamination with *P. vulgaris*.

1. INTRODUCTION

Proteus spp. is a Gram-negative rod-shaped bacterium that belongs to the Enterobacteriaceae family. It is classified in the family *Proteeae*, along with the genera *Morganella* and *Providencia* (Rozalski et al., 2012). *Proteus* genus members have a wide distribution in both the natural environment and gastrointestinal tracts of humans and animals (Hegazy, 2016). It is present in contaminated water, soil, and manure, where it has a significant function in breaking down organic material derived from animals (Mordi and Momoh, 2009). Proteus spp. bacteria are considered indicative of fecal contamination (Srinivasan et al., 2008).

Milk is consumed globally in a variety of forms as a staple diet for humans. When it is produced in the udder's alveoli, it is essentially a sterile fluid. However, at this step of production, microbial contamination may typically arise from various sources (Mennane et al., 2007). The contamination of raw milk at several key stages is caused by unhygienic activities during pre-milking udder preparation, inadequate hygiene of milk handlers, and unsatisfactory sanitation procedures related to milking and storage equipment. Milk is predominantly composed of water, in which a diverse array of elements such as vitamins, proteins, lipids, and carbs are dispersed. The abundance of nutrients in commercial milk, along with the production and processing methods, makes it vulnerable to contamination by several harmful microorganisms that can lead to human disorders. Consequently, milk is recognized as a possible source of transmitting disease-causing pathogens to people (Garedew et al., 2012).

Food-borne microbes are significant disease-causing agents that pose a threat to food safety and can lead to human illness on a global scale. This occurs when people consume food, particularly animal products that have been contaminated with live pathogens or their toxins (Abebe et al., 2020).

Proteus spp. presents a significant obstacle to both humans and animals globally, and it is widespread in many food and animal sources (Lei et al., 2014). There are already several strains of this bacterium that have developed resistance, which suggests a significant problem with food (Lei et al., 2016). Since the Proteus group of bacteria is responsible for many cases of food poisoning and more and more cases of foodborne infections, it is important to set up control programs and preventative measures to stop and deal with foodborne infections and poisoning (Ram et al., 2019). Antimicrobial-resistant bacteria can be transmitted from food to humans through cross-contamination (Lim et al., 2021). Human cases of Proteus are mostly contracted by the ingestion of contaminated food. Proteus typically spreads by the fecal-oral route, which involves the transmission of germs through contact with contaminated hands or objects that have come into contact with stool (Tonkić et al., 2010).

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Proteus vulgaris exhibits a wide range of methods for spreading and can therefore lead to infection in various parts of the body (Nita Pal et al., 2014). It is the primary cause of various opportunistic nosocomial infections, including those affecting the urinary system (Trivedi et al., 2015). It induces complex urinary tract infections (UTIs) more frequently than other uro-pathogens and contributes to the development of urinary stones. Additionally, it can cause infections in the respiratory tract, ear, nose, skin, burns, meningitis in newborns or babies, rheumatoid arthritis, and wound infections. Furthermore, it has the potential to cause gastroenteritis (Ebringer and Rashid, 2014).

The progressive rise of antibiotic resistance among clinical bacterial strains has emerged as a significant clinical concern (Adamus-Bialek et al., 2013). The rising concern lies in the evolution and dissemination of different mechanisms of antimicrobial resistance among prevalent human pathogenic members of Enterobacteriaceae, resulting in a reduction in accessible therapeutic alternatives (Boucher et al., 2009).

Nevertheless, there have been global reports of Proteus spp. that are resistant to many drugs (Singla et al., 2015). They possess the capacity to withstand various categories of antibiotics and are referred to as multi-antibiotic resistant (Dadheech et al., 2015).

This study aimed to investigate the occurrence of *Proteus vulgaris* in milk and dairy products, as well as the susceptibility of the isolated strains to antibiotics.

2. MATERIAL AND METHODS

2.1. 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.

2.2. Collection of samples:

A total of 150 milk and dairy product samples, (25 of each) comprising raw buffalo milk, Kariesh cheese, low-salt (Tallaga) cheese, Feta cheese, yoghurt, and ice cream were collected randomly from different sites in Benha city, Kalyobia government, Egypt at different time intervals Jan 2023 to Feb 2024 in the winter season. Every sample was meticulously placed in an individual plastic bag and swiftly conveyed to the Animal Health Research Institute (AHRI), Benha Branch in an insulated container filled with ice, guaranteeing absolute sterility without any extra postponement. The samples were promptly subjected to bacteriological testing to identify any contamination with *Proteus vulgaris* and evaluate their hygienic status.

2.3. Sample preparation (FDA, 2004):

Using aseptic techniques, a volume of 25 ml of milk (equal to 25 g of milk products) was measured and transferred into a sterile flask specifically built for homogenization. The flask contained 225 milliliters of sterile peptone water with a concentration of 0.1%. Subsequently, the resulting combination was modified to achieve an alkaline pH of 8.

2.4. Proteus species isolation:

The samples were cultivated in peptone water and incubated overnight at 37°C each tube was streaked onto Violet Red Bile Glucose agar (VRBG) plates and incubated at a temperature of 37°C for 24 hours. The obtained growth was subsequently transferred to MacConkey agar and placed in an incubator at 37°C for a further 24 hours. The pale colonies of non-lactose fermenting bacteria were then sub-cultured on, Xylose-lysin-deoxycholate (XLD) agar and blood agar to identify the occurrence of swarming.

The putative colonies were purified and subsequently inoculated onto nutrient agar tubes that were tilted for further identification. Subsequently, the specimens were grown on MacConkey agar.

2.5. Proteus species identification:

The suspected isolates of *Proteus species* were identified using the approach outlined by McFadden (2000). The isolates were analyzed through morphological examination, motility test, and a series of biochemical tests. These tests included indole, methyl red, Voges Proskauer, citrate utilization, urease, hydrogen sulfide production, gelatin liquefaction, nitrate reduction, Ornithine decarboxylase detection, L-lysine decarboxylase detection, Arginine decarboxylase detection, sugar fermentation, oxidase activity, and catalase activity.

2.6. PCR confirmation of P. vulgaris

The identification of the *ure*C gene of *P. vulgaris* was conducted as a biomarker to confirm the detection of these organisms according to Sammra et al. (2014)

2.6.1. DNA extraction from P. vulgaris isolates

One ml overnight incubated broth was centrifuged at a speed of 13000 rpm for 2 minutes at a temperature of 4 °C. The supernatant was subsequently discarded. The pellet was combined with 180 µL of a lysis buffer for Gram-negative bacteria. The lysis buffer contained 20 mM Tris-Hcl, 2 mM EDTA, 1.2% Triton X-100, and lysozyme at a concentration of 20 mg/ml. Subsequently, the blend was incubated for 30 minutes at a temperature of 37°C. Exactly, a combined volume of 200 µl of lysis solution and 20 µl of proteinase K were added and well mixed by vortexing to obtain a uniform suspension. The sample was incubated at a temperature of 56 °C and regularly vortexed until the cells were completely lysed, which took approximately 30 minutes. Furthermore, 20 µl of Rnase A solution was added, well mixed by vortexing, and thereafter incubated for 10 minutes at room temperature. Afterward, 400 µl of a solution containing 50% ethanol was added and well mixed using vortexing. The produced lysate was transferred to a GeneJET Genomic DNA Purification Column, which was then placed within a collecting tube. The column was centrifuged with a force of 6000 times the acceleration due to gravity for 1 minute. The collecting tube with the fluid that successfully passed through was disposed of. The GeneJET Genomic DNA purification column was placed into a new 2 ml collection tube. Alternatively, 500 µl of Wash Buffer I (including ethanol) were added and centrifuged at a force of 8000 times the acceleration due to gravity for 1 minute. The waste liquid that passed through the system was reintroduced into the collection tube upon replacing the purification column. Furthermore, 500 µl of Wash Buffer II, which contains ethanol, was added to the GeneJET Genomic DNA Purification Column. The column was then centrifuged at the highest speed (12000 ×g) for 3 minutes. The collection tube containing the solution that has passed through is disposed of, and the GeneJET Genomic DNA Purification Column is moved to a sterile 1.5 ml microcentrifuge tube. To extract the genomic DNA, precisely 200 µL of Elution Buffer was added to the middle portion of the GeneJET Genomic DNA Purification Column membrane. The DNA was incubated at room temperature for 2 minutes and then centrifuged at a force of 8000 times the acceleration due to gravity for 1 minute.

The purification column was disposed. The pure DNA should be either used immediately in subsequent procedures or stored at a temperature of -20°C.

2.6.2. The PCR reaction for the tested gene was performed A total volume of 25 µl reaction mixture containing 2 µl of each forward and reverse primer (10 pmol), 20 µl of sterile water and 1 µl of DNA-mixture using the following program: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles. The cycle comprised denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Subsequently, the amplified samples underwent electrophoresis on a 1.5% agarose gel at a voltage of 80 V for 90 minutes (Kamil and Jarjes, 2019). Afterward, the samples were examined under ultraviolet (UV) light following treatment with ethidium bromide. A 100-base pair DNA ladder was used as a molecular size marker.

2.7. Antibiotic resistance of isolated bacteria (Antibiogram):

The antimicrobial susceptibility of the isolated *Proteus species* was evaluated using the disc diffusion method as outlined by Al-Kharousi et al. (2019). Antimicrobial discs concentrations (Oxoid Limited, Basingstoke, Hampshire, UK). The bacterial culture was uniformly dispersed across the whole surface of the nutrient agar plate. Consequently, the antibiotic discs were placed on the surface of the diseased plate. then the plate was placed in the incubator at 37°C for 24 hours. The plate was subsequently analyzed to ascertain the presence of bacterial proliferation in the vicinity of the antibiotic discs.

The isolates were subjected to the disk diffusion test against antimicrobial discs (Bio analyses); Amoxicillin (25µg), Amikacin (AK) 30 ug, Levofloxacin (Le) 5µg, Oxytetracycline (30µg), gentamicin (10µg), Erythromycin (15µg), Trimethoprim Sulfamethoxazole (25µg), Ciprofloxacin (CP) 5Ug, Gentamicin (CN) 10 ug, Cefotaxime (Ctx) 30 ug, Ampicillin (AM) 10 ug, Meropenem (M) 20\10ug , Imipenem (IPM) 10ug, Clindamycin (CL) 2UG According to *(Markey et al., 2013)* and the inhibition zones were interpreted according to CLSI (2020).

Table 1 Antimicrobial Inhibition zone diameter of *Proteus vulgaris* strains (n=9).

Antimicrobial agent	S		I		R	
	Inhibition zone diameter		Inhibition zone diameter		Inhibition zone diameter	
Clindamycin (CL) 2UG	≥21mm		15-20mm		14≥mm	
Erythromycin (E) 15ug	≥21mm		16-20mm		15≥mm	
Trimethoprim sulfamethoxazole (SXT) 1.25\23.75ug	≥16mm		11-15mm		10≥mm	
Oxytetracycline (T) 30ug	≥15mm		12-14		11≥mm	
Ampicillin (AM) 10ug	≥17mm		14-16mm		13≥	
Penicillin-G (P) 10units	≥15mm				14≥	
Amoxicillin (AMX) 25UG	3	33.3	2	22.2	4	44.4
Levofloxacin (Le) 5mcg	≥21mm		17-20mm		16≥ mm	
Ciprofloxacin (CP) 5Ug	≥26mm		22-25mm		21≥ mm	
Gentamicin (CN) 10ug	≥15mm		13-14MM		12≥ mm	
Cefotaxime (Ctx) 30ug	≥26mm		23-25mm		22≥ mm	
Amikacin (AK) 30 ug	≥18mm		14-17mm		13≥ mm	
Cefepime (FEP) 30ug	≥18mm		15-17mm		14≥ mm	
Meropenem (M) 20\10ug	≥18mm		17-15mm		14≥	
Imipenem (IPM) 10ug	≥23mm		20-22mm		19≥	

3. RESULTS

In the current study, the overall prevalence of *P. vulgaris* in examined milk and dairy products was 6% (9 out of 150 samples). The prevalence rates of *P. vulgaris* were 12%, 16%, 4%, 0%, 4%, and 0% in the examined samples of raw buffalo milk, Kariesh cheese, low-salt (Tallaga) cheese, Feta cheese, yoghurt, and ice cream, respectively (Fig. 1).

Agarose gel electrophoresis of PCR of the *UreC* gene (263 bp) was employed for confirmation of *P. vulgaris*. This gene was detected in all *P. vulgaris* isolates (9 strains) (Fig. 2).

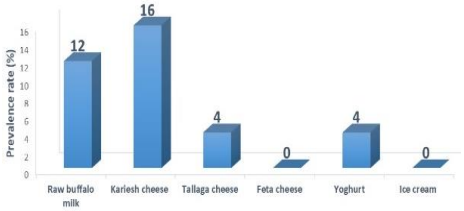

Fig. 1 Prevalence rates (%) of *Proteus vulgaris* in the examined raw milk and dairy product samples

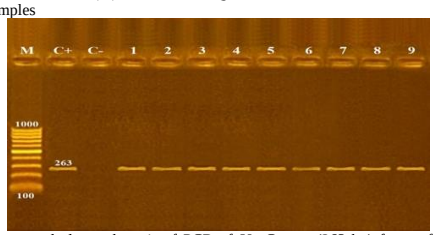

Fig. 2 Agarose gel electrophoresis of PCR of *UreC* gene (263 bp) for confirmation of *Proteus vulgaris*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *P. vulgaris* for *UreC* gene. Lane C-: Control negative. Lanes from 1 to 9: Positive strains for *UreC* gene.

Proteus vulgaris isolates showed resistance to more than one antibiotic. All *Proteus vulgaris* isolates showed complete resistance to erythromycin and clindamycin . Also, 77.8 % of *Proteus vulgaris* isolates were resistant to trimethoprim sulfamethoxazole. The lowest degree of resistance was detected to ceftazidime, meropenem, and imipenem at 11.1% for each. (Table 2).

Table 2 Antimicrobial susceptibility of *Proteus vulgaris* strains (n=9).

Antimicrobial agent	S		I		R	
	NO	%	NO	%	NO	%
Clindamycin	-	-	-	-	9	100
Erythromycin	-	-	-	-	9	100
Trimethoprim Sulfamethoxazole	-	-	2	22.2	7	77.8
Oxytetracycline	1	11.1	3	33.3	5	55.6
Ampicillin	2	22.2	2	22.2	5	55.6
Penicillin-G	4	44.4	-	-	5	55.6
Amoxicillin	3	33.3	2	22.2	4	44.4
Levofloxacin	4	44.4	1	11.1	4	44.4
Ciprofloxacin	5	55.6	1	11.1	3	33.3
Gentamicin	6	66.7	-	-	3	33.3
Cefotaxime	5	55.6	2	22.2	2	22.2
Amikacin	6	66.7	1	11.1	2	22.2
Cefepime	6	66.7	1	11.1	2	22.2
Meropenem	7	77.8	1	11.1	1	11.1
Imipenem	8	88.9	-	-	1	11.1

4. DISCUSSION

Contamination of raw milk and dairy products with *Proteus spp.*, particularly *P. vulgaris* is indicative of their fecal contamination. In this direction, raw milk and kariesh cheese in this study had the highest prevalence of *P. vulgaris.* This could be attributed to the cross-contamination of the raw milk from the animal's udder or milker's hands during the milking process or the use of contaminated utensils for the collection of milk. The

manufacturing process of kariesh cheese involves direct exposure of the cheese to the open air and can be easily contaminated by the flies or air at the market or during their preparation (Elafify et al., 2019). This finding surpasses the findings of Syed (2013), who reported that 18% of milk and milk product samples (such as curd and ice cream) were contaminated with *Proteus spp*. Additionally, Sobeih et al. (2020) isolated *P. vulgaris* from 13.10% of raw milk samples and 4.76% of ice cream samples. Enterobacteriaceae, particularly *Proteus spp.* was isolated from raw milk and kariesh cheese retailed in Menofiya Governorates at comparable rates (El Refaey et al., 2023). However, Awad et al. (2005) recorded decreased isolation rates, indicating that Proteus spp. was not found in the raw milk samples analyzed. However, it was detected in 8% of the Damietta cheese samples and 4% of the kariesh cheese samples.

The abuse and uncontrolled usage of antimicrobials during livestock production have resulted in the development of drug resistance among foodborne pathogens. In this regard, the findings of the present investigation reported that all *Proteus vulgaris* isolates showed resistance to erythromycin and clindamycin and 77.8 % of *Proteus vulgaris* isolates were resistant to sulphamethoxazol. Such findings are consistent with the reports of AL-Ta'ee (2002), who observed that the majority of Proteus isolates exhibit a high level of resistance to tetracycline, with rates of 96%. Akerele et al. (2001) discovered that *Proteus spp.* exhibit a sensitivity rate of 72.1% to tetracycline. In Ajmer Region, India, Dadheech et al. (2015) observed that *Proteus spp.* showed complete resistance to tetracycline. The data observed can be related to the widespread use of tetracycline in routine prophylaxis and chemotherapy for livestock management in Nigeria (Aliyu et al., 2019). In a study conducted by Fallah et al (2019) in Iran, it was found that the rate of resistance to ciprofloxacin, ceftriaxone, and imipenem was higher than what was observed in our study. This suggests that the level of resistance to these antibiotics has grown among *P. vulgaris* isolates.

5. CONCLUSIONS

In sight of the previous facts, it is highly recommended to adopt strict hygienic measures during the manufacturing process of dairy products to avoid contamination of such products with Proteus spp., particularly, *P. vulgaris*.

This investigation successfully isolated *P. vulgaris* from raw milk and dairy products including Kariesh cheese and yoghurt, sold in retail, with varying rates of occurrence in Benha city. The isolated strains exhibited significant antibiotic resistance against erythromycin and clindamycin. Consequently, it is imperative to adhere to stringent hygiene protocols when collecting raw milk at retail and throughout the production of dairy products to prevent the contamination of these products with enteric bacteria.

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