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Isolation and Identification of *proteus spp* from hatcheries and table eggs in Kerbala City

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***Abstract*—** This study set out to measure the frequency of Proteus spp. in hatcher settings gathered in Karbala City, as well as to identify and isolate these species. Bacteria belonging to the Magnoliaceae family, which are peritrichously flagellated, are distinguishable from other members of the order Enterobacterales by the multicellular differentiation phenomenon, which causes them to proliferate in intense and spectacular swarms on solid substrates. Three hundred samples were collected from different hatcheries in the city of Karbala, and culture tests such as (blood agar, MacConkey agar and XLD) and chemical tests such as (G stain, Catalase Oxidase test, Indole test, Cimone Citate test, Triple sugar ion (TSI) agar test and Urase production) were performed. Isolation was confirmed using the VITEK apparatus. The culture and chemical results showed the presence of bacteria Proteus spp .Out of the 300 isolates that were examined, the following categories of samples tested positive (14% of the total): newly 36.6% of hatched chicks, 13.3% of workers, 6.6% of doors, 0% of trays, 0% of incubators, 40% of chicken droppings, 0% of cardboard envelopes, 33.3% of the floor, 26.6% of fans, 20% of ventilation openings, and eggs and other developmental stages (0%) in conclusion P. mirabilis exhibits versatility bacterium recognized as a significant opportunistic uropathogen, particularly known for causing complicated in chicken hatcheries.

***Keywords* —** Proteus mirabilis, hatcheries, bacterial isolation.

INTRODUCTION

The genus Proteus belongs to the Morganellaceae family within the order Enterobacterales, which includes Gram-negative Bacillus, and to the Tribe known as Protease, which includes 3 genera (Proteus, Morganelli and Providencia spp (1). Proteus spp. bacteria are peritrichously flagellated members of Morganellaceae family distinguished from the other representatives of the order Enterobacterales by intensive and spectacular swarming growth on solid media as a result of the multicellular differentiation phenomenon. (2) The rod-shaped, facultatively anaerobic, non-capsulated, non-spore-forming, motile, and urease-splitting bacteria known as Proteus can be described by its Gram-negative characteristics. (3) Appropriate environmental circumstances allow it to produce a range of opportunistic nosocomial infections; it is most commonly seen in nature. (4)These bacilli can be found in soil and water, as well as in a wide variety of animals, both wild and domestic. An additional portion of the human population has these bacteria as part of their native fecal microbiome. However, opportunistic microorganisms like Proteus spp. can infect a wide variety of organs, including the urinary tract and wounds, and can even cause bacteremia and other infections in those with impaired immune systems. (5).The character Proteus, who has the capacity to change his shape and undergo unending transformations, was named Proteus in Homer's Odyssey. Proteus vulgaris and Proteus mirabilis were the two species of the genus that were described by Hauser. (7) A shell-embryo-pathogen horizontal transmission that causes inflammation and infection. From before fertilization until after the Poultry is born, germs like Salmonella Enteritidis, Klebsiella, Micrococcus, Proteus, Pseudomonas, Staphylococcus spp., and Escherichia coli can be present. (8,9) Nevertheless, several egg-borne diseases, some of which are extremely zoonotic, might be introduced to them through their contact with surroundings such as faucal matter and nesting materials. Numerous worldwide epidemics linked to eating eggs, caused by germs found in eggs and their shells, demonstrate that this is a serious problem everywhere. It is worth mentioning that eggshells and egg contents have been shown to contain food-borne diseases like Salmonella, Proteus, and some gram-positive bacteria, such as Staphylococcus aureus (10).Contamination of the egg shell the egg shell serves as a physical, physiological, and immunological barrier that controls the embryo's health and development by making it structurally impermeable to pathogens and by expressing proteins that respond to infections with an antimicrobial response (11,12). The aims of study to investigate the occurrence of Proteus spp. in hatchery environments and hatching eggs using both conventional bacteriological techniques

MATERIALS AND MTHODS

A total of 300 hundred sample was collected from Karbala city consist of 100 samples from Al- Furat Al-Awsat

Hatchery, 100 samples from Al Rahma Hatchery and 100 samples from Sayyid Jalal Hatchery. Sample Classification per Site (20 samples/site), Eggs samples were collected from September 2025 to November 2025 and different incubation ages of developmental stages. Hatchery Environment Surfaces (doors, floors) Equipment (trays, incubators) Air (fans, ventilation openings) Packaging materials (cardboard boxes).

Samples were collected using a refrigerated box and sent to the Public Health Laboratory at the College of Veterinary Medicine, University of Karbala for culture and chemical tests according to(13). Samples were cultured using culture media (blood agar, MacConkey agar and XLD) and chemical tests were performed (G stain, Catalase Oxidase test, Indole test, Cimone Citate test, Triple sugar ion (TSI) agar test and Urase production) The isolates were confirmed using the Vitek All procedures in this study were reviewed and approved by the local Committee on Animal Use and Care, College of Veterinary device after the turbidity of the isolates was performed using the 0.5 MacFarland.

Experimental design

Sample collection includes isolation and identification proteus spp from hatcher egg. A total of 300 sample from hatcheries and hatcheries egg samples (Al-Furat Al-Awsat, Sayyid Jalal &. Al-rahma) were collected from different regions in Karbala City, Iraq. It was obtained from 3 places in the city of Karbala during the period from (September 2025-november 2025). Samples were transported under sterile conditions via transport medium (Nutrient broth & transport media) by ice boxes under refrigeration to the laboratory within 24 hours for further analysis. The samples were categorized into two groups: (egg in different stage &environment) in each region was take 100 samples collection from (Al-Furat Al-Awsat Hatchery 100 sample, al Rahma Hatchery 100 sample and Sayyid Jalal Al-Sherfy Hatchery 100). Each 100-sample divided in (Different incubation eggs varying developmental stages and, from hatchery environment: surfaces (doors, floors) equipment (trays, incubators) air (fans, ventilation openings) A total of 300 samples from hatcheries, bacterial isolation & identification by Biochemical tests.

RESULT AND DISCUSSION

Isolation and Identification of Proteus spp

Table 1. Number and percentage of Bacterial ontamination.

no	Sample Type	details	no. of Samples (n)	Positive Samples (n)	Positive Percentage %	negative Samples (n)	negative Percentage %
1	Egg in different stage	**1 day**	30	-	0%	30	100%
		9 days	30	-	0%	30	100%
		18 days	30	-	0%	30	100%
2	**Surface**	**Floor**	15	5	33.3 %	10	66.6 %
		Doors	15	1	6.6 %	14	93.3 %
3	**Trays & incubators**	**Trays**	30	-	0 %	30	100%
		Incubator	15	-	0%	15	100%
4	**Air**	**Fans**	30	8	26.6 %	22	73.3 %
		Ventilation opening	15	3	20%	12	80%
5	**Newly hatched chicks are weak**		30	11	36.6 %	19	63.3 %
6	**worker**		15	2	13.3 %	13	86.6 %
7	**chicken droppings**		30	12	40%	18	60%
8	**Cardboard envelopes**		15	-	0%	15	100%
	Total		300	42	14%	258	86%
	Statistical analysis Chi-square test		**X2= 21.577 Df=1 P< 0.001 significant**				

The results showed significant differences ($P < 0.05$), indicating that chicken droppings had the highest contamination rate with Proteus spp. (40%), followed by weak chicks at a rate of 36%. Gram-Stain Reagents All of it was done in accordance with the directions given by.

Conventional Identifications of bacterial isolation: Gram Stain Solutions Those were prepared and used according to the method recommended by. (24). The shape and arrangement of cells, as well as the ability to distinguish between Gram-negative and Gram-positive bacteria, were investigated using this method. Color, shape, gram-stain reaction, cell arrangement, and rearranging cells were all part of the microscopic analysis that proved the bacterial isolates' morphology, Oder, pigment, size, swarming, and motility. Because it G- (takes up pink color from safranin and its cell membrane is weak, it cannot preserve crystal violet, and four regent crystals—alcohol, iodine, safranine red, and proteus—appear pink or red.

Colonies developed on MacConkey agar from Proteus spp. were found to be colorless or pale, which is in line with the fact that this bacterium is not known to ferment lactose, according to the current study. The neutral red indicator in the medium maintained its original color without acid production due to the lack of lactose fermentation. As a result, the surrounding agar maintained its pinkish hue without the precipitation of bile salts. The colonies were typically medium sized, circular, smooth, and slightly moist, with entire margins and a slightly convex elevation (figure 1.A). However, the presence of bile salts and crystal violet in MacConkey agar prevented it from exhibiting its characteristic swarming motility, in contrast to its behavior on non-selective media like blood agar or nutrition agar (figure 1.B). Microscopic examination revealed Gram-negative, rod-shaped cells, which were highly motile owing to the presence of peritrichous flagella, a feature that contributes to their spreading ability on suitable culture media.

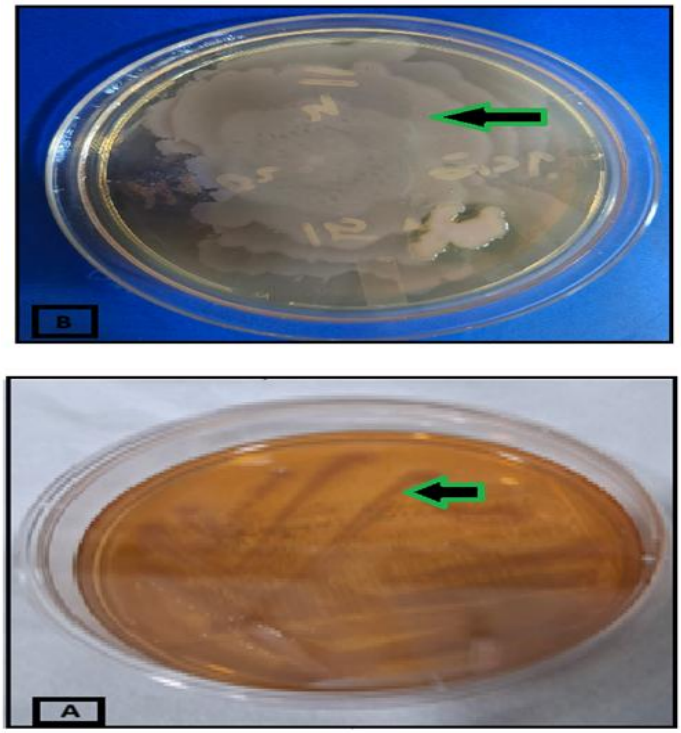

Figure 1.A. Proteus mirabilis cultured on MacConkey agar. Colorless, non lactose fermenting colonies (indicated by arrows) were observed after 24 h of incubation at 37 °C.; Proteus mirabilis cultured on blood agar. Characteristic swarming motility (indicated by arrow) was observed, with thin, spreading growth radiating from the central inoculation site. Beta hemolysis was also evident as clear zones surrounding the colonies.

The growth patterns of Proteus mirabilis on both selected and non-selective medium were compatible with its known taxonomic traits, as shown in this study's conventional identification. Colonies of P. mirabilis on MacConkey agar were found to be colorless or pale because the bacteria could not digest lactose. The absence of the β-galactosidase enzyme, which is present in non-lactose-fermenting Enterobacteriaceae, prevents the organism from converting lactose into fermentable sugars and producing acid, which would change the color of the neutral red indicator in the medium (14). Instead of the typical spreading growth seen on non-selective media, discrete colonies were observed on MacConkey agar due to the presence of bile salts and crystal violet, which inhibit the growth of Gram positive bacteria and suppress the swarming motility of Proteus species. Antiswarming substances like crystal violet and bile salts have a well-documented inhibitory effect; they do not harm the organism's survival but do limit its migration to the surface (15), The Gram-negative rod-shaped cells with peritrichous flagella that were seen in the isolated colonies under the microscope were very motile; this finding is in agreement with the genus description from Bergey's Manual of Systematics of Archaea and Bacteria (16).

On blood agar, P. mirabilis exhibited characteristic swarming motility, a phenomenon first described by (15). Radiating from the center of the inoculation site, thin, spreading growth formed concentric zones of bacterial movement, indicating swarming. Coordinated population movement over solid surfaces, enhanced flagella synthesis, and cellular elongation are all intricate steps in the swarming differentiation process in Proteus species. Moreover, beta-hemolysis was noted in the vicinity of the colonies in this investigation, suggesting the generation of hemolysins—noted as critical virulence factors in P. mirabilis infections (17,22).

In the present study, it was found that when Proteus mirabilis was cultured on XLD agar, black centered colonies were consistently produced after 24 h of incubation at 37 °C. The colonies appeared translucent with a black center, and no surrounding color change indicative of xylose or lysine metabolism was observed. The black pigmentation was attributed to the production of hydrogen sulfide (H_2S), which reacted with ferric ammonium citrate present in the medium to form ferric sulfide precipitates. The deoxycholate content of the medium effectively inhibited swarming motility, allowing discrete colonies to be visualized figure (2).

Figure 2. Proteus mirabilis cultured on XLD agar. Black centered colonies (indicated by arrows) were observed after 24 h of incubation at 37 °C, indicating hydrogen sulfide (H_2S) production. Swarming motility was inhibited by the deoxycholate content of the medium, allowing discrete colony formation.

The biochemical profile of P. mirabilis, which is known to generate H2S from thiosulfate or other sulfur-containing substrates, was compatible with the finding of black colonies on XLD agar. The initial purpose of XLD agar was to isolate Shigella and Salmonella species; it contains ferric ammonium citrate as an indicator and sodium thiosulfate as a substrate for H_2S generation (16). The presence of black precipitates inside the colonies, a byproduct of the interaction between H_2S and ferric ions that produces insoluble ferric sulfide, proved that H_2S was produced in the current investigation (18).

Bile salts (deoxycholate) and crystal violet were thought to be responsible for the suppression of swarming motility on XLD agar, which was comparable to the impact seen on MacConkey agar. Because of this quality, it was easy to separate the colonies and see the H_2S response up close. Because it selectively isolates Gram-negative enteric pathogens and distinguishes them according to carbohydrate fermentation and H_2S generation, XLD agar is a useful tool in clinical microbiology for specimens that could have mixed flora (19).

In the present study, it was found that the bacterial isolates exhibited a consistent set of biochemical characteristics, confirming their identification as Proteus mirabilis. Gram staining revealed pink, rod-shaped cells, confirming the isolates were Gram-negative bacilli. The catalase test yielded a positive result, indicated by immediate bubble formation upon exposure to hydrogen peroxide. Conversely, the oxidase test produced a negative result, as no color change was observed. The indole test was negative, as evidenced by the retention of a yellow or brown ring at the surface of the Kovac's reagent, with no red coloration. The citrate utilization test was positive, demonstrated by the appearance of intense blue coloration on Simmons citrate agar, indicative of growth and alkaline shift. On triple sugar iron (TSI) agar, the isolates produced an alkaline slant (K), an acid butt (A), and both hydrogen sulfide (H_2S) production and gas generation were observed. Finally, the urease test was strongly positive, as indicated by a rapid color change from yellow to pink or red, signifying urea hydrolysis table (1).

Table 2. Biochemical test of *Proteus mirabilis*

No	Biochemical test	Test
1	G-stain	(Pink Rod shape)
2	Catalase	+
3	Oxidase test	-
4	Indole test	(+) red or pink ring at surface p.valgarus. (-) yellow or brown ring at surface p.mirballus
5	Cimon Citate test	Growth Appear as Intense Blue Color = (+) growth non change = (-) negative
6	Triple sugar iron (TSI) agar test	(K/A, H_2S +, Gas+)
7	Urase production	(+)

The biochemical profile of the isolates as a whole was in agreement with the known features of P. mirabilis, a member of the family Enterobacteriaceae. One of the main characteristics that distinguishes the genus Proteus is its Gram-negative rod shape (20). The characteristic pattern of the Enterobacteriaceae family—a positive catalase response together with a negative oxidase reaction—helps to distinguish them from other Gram-negative bacteria, such Pseudomonas species, which are oxidase (21) figure 3(A and B). One important biochemical indicator that differentiates P. mirabilis from Proteus vulgaris is the absence of indole in the test. A negative reaction occurs because P. mirabilis lacks the enzyme tryptophanase, which is necessary for P. vulgaris to hydrolyze tryptophan and produce indole. The capacity of P. mirabilis to use citrate as its only carbon source is demonstrated by the positive citrate utilization test, which is characterized by growth with a bright blue hue. This differentiation is crucial for correct speciation. This feature, in conjunction with the other tests, distinguishes it from other types of enteric bacteria, such as the citrate-negative Escherichia coli (22).

The response that was seen on TSI agar, which is acid butt/alkaline slant with H_2S and gas generation, is characteristic of P. mirabilis. The bacterium uses peptone to create an alkaline slant, as it ferments glucose (resulting in an acid butt) but not lactose or sucrose. One distinctive trait is the creation of hydrogen sulfide, which, when combined with ferric ions, forms a black precipitate. (23) An important component of P. mirabilis's pathogenicity is the presence of a robust positive urease test. Ammonia is produced by the fast breakdown of urea by the urease enzyme, which causes the pH to rise. Kidney stones and catheter encrustation can develop when struvite and apatite crystals precipitate as a result of this alkalinization in the setting of a urinary tract infection (22).

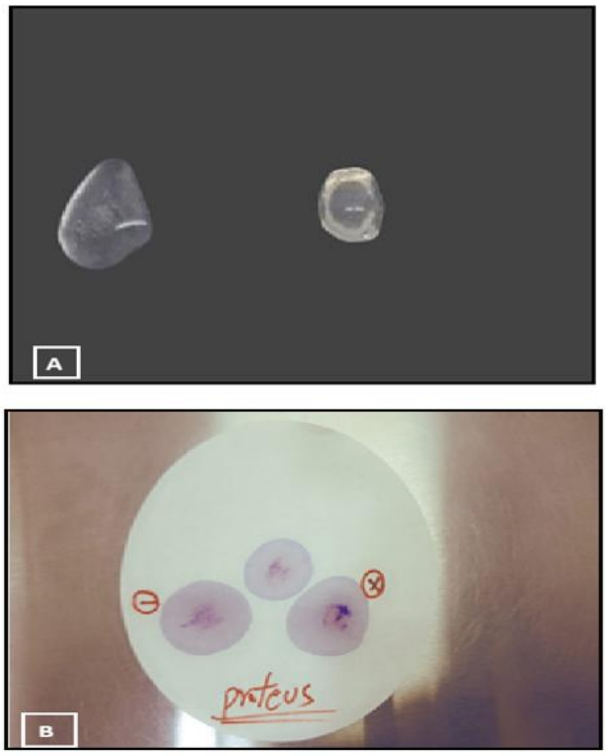

Figure 4 A. represented coagulase positive test of proteus mirabilis and **B:** represented Oxidase positie test for proteus mirabilis.

Following phenotypic characterization, all isolates presumptively identified as Proteus mirabilis were subjected to confirmation using the Vitek-2 automated system (bioMérieux, Marcy l'Étoile, France). The testing procedure was performed according to the manufacturer's protocol, utilizing the identification card (GN ID card) designed for Gram-negative bacilli. The results are presented .The Vitek-2 system identified all tested isolates as Proteus mirabilis with a probability score of 90% or higher, confirming the species identity. Statistical analysis was conducted to evaluate the concordance between conventional biochemical identification and Vitek-2 confirmation, and a high level of agreement.

CONCLUSION

The results of this work demonstrated the conventional phenotypic tests (Gram stain, catalase, oxidase, indole, citrate, TSI, urease) together with Vitek 2 automated identification confirmed that all isolates were Proteus mirabilis. Molecular identification by 16S rRNA gene amplification and

phylogenetic analysis further supported this identification, with the three local strains (accession numbers P2025505, P2025506, P2025507) forming a well supported clade (98% bootstrap) with reference P. mirabilis sequences from various geographic origins. The epidemiological investigation revealed an overall P. mirabilis prevalence of 14% in the hatchery environment. The highest contamination rates were found in chicken droppings (40.0%), newly hatched weak chicks (36.6%), floor surfaces (33.3%), and air fans (26.6%). No isolates were recovered from egg samples at any incubation stage, suggesting that transmission occurs primarily after hatching via environmental reservoirs rather than through vertical transmission.

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N/A

Conflict of Interest

The authors declare no conflict of interest.

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