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Molecular Detection of Virulence Genes in *Pseudomonas aeruginosa* Isolated from Different Clinical Specimens of Cows in Baqubah, Iraq

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Abstract

Background: The bacterium *Pseudomonas aeruginosa* causes opportunistic infections in animals and humans. Many of the infections are caused by several of their virulence factors, including phospholipase C, Exoenzyme S, and Exotoxin A.

Aim: The goal of the study was to isolate *Pseudomonas aeruginosa* in clinical cow case samples in Baquba, Iraq and to determine the prevalence of the virulence associated genes, *toxA*, *exoS*, and *plcH*.

Methods: A total of 168 different samples were collected from various clinical cases, including 53 urine samples, 53 ear samples and 62 milk obtained from infected cows in Baqubah city Samples were taken in hopes of positive bacterial infections. Identification of the bacterial isolate was done through culture, biochemical tests, and the use of the VITEK 2 system. The presence of the genes *toxA*, *exoS*, and *plcH*, were examined through polymerase chain reaction (PCR) with the use of specific primers

Results: Ten samples collected were positive for *P. aeruginosa*. Most positive samples were urine samples, and the least positive samples were ear swabs, while milk samples were negative.. Seven of them produced positive colonies on cetrimide agar.. 9 of 10 positive results (90%) for the presence of the gene *toxA*. displays 5 of 10 (50%) positive results for the presence of the gene *exoS*. 7 of 10 (70%) were positive for the *plcH* gene.

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Conclusions: Urine samples were the most common positive samples for *P. aeruginosa*. VITEK 2 was the most helpful system in providing identification. The virulence genes that were discovered are indicative of bovine infections. The specific and rapid detection of *Pseudomonas aeruginosa* through PCR allowed for the detection of the virulence genes.

Keywords: *Pseudomonas aeruginosa*, virulence genes, *toxA*, *exoS*, *plcH*

Introduction

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, opportunistic pathogen that leads to a large number of hospital-acquired infections (Pang *et al.*, 2019). It is found everywhere, whether in soil, water, or in living hosts. Its role in infection is attributed to its ability to adapt to different environments (Krell & Matilla, 2024). *Pseudomonas aeruginosa* is considered to be a major agent of infection in humans, animals, and plants, and poses a particular threat to the immunocompromised and people with chronic diseases (Milivojevic *et al.*, 2018). Veterinary *P. aeruginosa* is often found to be the source of infection in both livestock and pets. In livestock, this includes dairy cattle and goat mastitis, while in pets, it is responsible for urinary tract infections and external ear infections. Hemorrhagic pneumonia in foxes and minks, and endometritis in mares (Shahat *et al.*, 2019). In a recent study in northeastern Poland, 98 *P. aeruginosa* isolates were analysed from various species of animals, including dogs, cats, ruminants, and birds. It was found that all isolates had the *pelA* gene, with the *pldA* (24%) and *exoU* (36%) showing the lowest prevalence (Ruminant Study, 2024).

In cattle, *P. aeruginosa* mastitis mainly occurs in dry cows or those who have just calved. Isolation of *P. aeruginosa* in bovine milk samples in Iraq have been documented. Jasim *et al.* (2024) identified *P. aeruginosa* in 60% of bovine milk samples and 73.33% of nasal discharge samples from cows in Basra province, and all the isolates were multi-drug resistant (MDR) to antibiotics, including imipenem. In addition, Saeed *et al.* (2023) reported 11 raw milk isolates in Diwaniya and Al-Najaf and 16S rRNA positive samples from 70 of the milk samples. In Basrah, Al-Tememe and Abbas (2022) were also able to isolate *P. aeruginosa* from ear swabs of infected ruminants. The organism's fecal high carriage rate may lead to the contamination of farm water sources and certain pyocin types in the udder, gut or water may aid in the spread of the organism

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(Shahat *et al.*, 2019). Antimicrobial resistance as a result of the inappropriate use of antibiotics in human medicine and animal husbandry has become a serious threat to use of the available antibiotics and this extremely limits the treatment options in the infected individuals (Shahat *et al.*, 2019).

P. aeruginosa has many virulence factors, which explains its pathogenicity. Exotoxin A, which comes from the *toxA* gene, causes cell death due to the ADP-ribosylation of elongation factor-2, which halts protein synthesis (Al-Muthanna study, 2025). Another virulence factor, Exoenzyme S, comes from the *exoS* gene, and is a type III secretion system effector disrupting host-cell signaling and the cytoskeleton (Özsoy *et al.*, 2024). Phospholipase C is coded by *plcH* and causes tissue damage and hemolysis due to the degradation of phospholipids (Al-Muthanna study, 2025). New research has confirmed the presence of virulence genes for *P. aeruginosa* from multiple sources. For example, Taha (2025) studied Iraqi fish and found many *P. aeruginosa* isolates with the *toxA* gene. Furthermore, Zghair and Mathree (2025) published findings on the presence of *exoS* and additional virulence genes in *P. aeruginosa* isolates from both humans and chickens in Wasit, Iraq. Given this, the objectives of this research are to: (1) isolate and identify *P. aeruginosa* from urine, ear swab, and milk samples of cows from Baqubah city, Iraq, (2) The VITEK 2 system was used to identify the bacteria., and (3) Using specific primers and optimized thermal cycling conditions to detect the virulence genes toxA, exoS, and plcH by polymerase chain reaction.

Materials and Methods

Between 1st September 2025 and 1st of February 2026, A total of 168 different samples were collected from various clinical cases, including 53 urine samples , 53 ear samples and 62 milk obtained from infected cows in Baqubah city .ear samples were collected with sterile cotton swabs and placed into sterile test tubes containing 5 mL of brain heart infusion broth. Milk and urine swabs were also collected into sterile containers. All samples were properly collected and transported to the laboratory within two hours of collection. All samples were collected in an aseptic condition. Bacteriological analyses of the samples were done at the University of Diyala, Iraq, at the Department of Veterinary Microbiology, Faculty of Veterinary Medicine.

Bacterial Isolation and Identification

All samples were placed on MacConkey agar media and incubated at 37°C for 18–24 hours. Samples were critically and carefully selected in the nearby areas of the samples after the incubation and watching the colonies for colonial morphology (Shaymaa and Aida ,2023). Samples were selected to have those that were pale in color, colonies that did not have any lactose fermentation, and colonies that had smell of grapes or odor like dust for the purpose of

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studies (Jasim *et al.*, 2024). In preliminary identification of the samples were the Gram stain, and for the other samples were oxidase, and catalase test, were tested and also the samples were grown on cetrimide agar, that was selective for *P. aeruginosa* ((Hassoon *et al*. 2024).). In cetrimide agar, *P. aeruginosa* were grown, and the colonies were produced a fluorescent yellowish green colored pyoverdine, and there were bluish green colored colonies that were produced due to pyocyanin pigment, and it was like the what is presented in Figure 1. The confirmatory of the identification was done using VITEK 2 Compact Automated System (bioMérieux, France) basing on the instructions of the manufacturer. (Ahmed *et al*., 2025).

DNA Extraction

For the DNA extraction, the manufacturer's procedures were followed. The extraction kit used was the FavorPrep Total DNA Mini Kit from FavorGen, Korea. Purity and concentration were measured with a NanoDrop One UV-Vis spectrophotometer from Thermo Scientific, USA. The DNA was then stored at -20 degrees Celsius until it was used for the PCR amplification.

PCR Amplification of Virulence Genes

The target for the PCR was to identify three virulence genes, such as exotoxin A (*toxA*), exoenzyme S (*exoS*), and hemolytic phospholipase C (*plcH*). In Table 1,

Table (1): Primer sequences used in this study

Gene	Primer	Sequence (5' → 3')	Tm (°C)	GC%	Size (bp)
plcH	F	GCACGTGGTCATCCTAGTGC	63.6	60	608
	R	TCCGTAGGCGTGCAGCTAC	63.7	63	
toxA	F	ACCTGATACCCACTTGGATC	59.8	50	745
	R	TAGATCTTGCCTTCCCAGGTA	60.2	48	
exoS	F	TGCATATTCAATCGCTTCAG	53.7	40	643
	R	CTCATTACCTGATGCAACTG	54.8	45	

The PCR reaction mixture (25 µL total) consisted of 12.5 µL of PCR master mix, 9 µL of distilled water, 1 µL of each primer (10 pmol/µL), and 1.5 µL of DNA template. Thermal cycling conditions for each gene in the initial denaturation, denaturation (35), annealing (35), and extension (35) steps, final extension, and temperatures are provided in Table 2.

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Table (2): PCR for all genes thermal cycling conditions

Gene	Initial Denaturation	Denaturation (35 cycles)	Annealing (35 cycles)	Extension (35 cycles)	Final Extension
toxA	95°C/3 min	95°C/30 sec	58°C/30 sec	72°C/30 sec	72°C/30 min
exoS	95°C/3 min	95°C/30 sec	57°C/30 sec	72°C/30 sec	72°C/30 min
plcH	95°C/3 min	95°C/30 sec	63°C/30 sec	72°C/30 sec	72°C/30 min

After amplification, PCR products were analyzed by electrophoresis on 1.5% agarose gels with ethidium bromide staining, followed by UV light visualization. DNA ladder 100–1500 bp was used as a size marker. Results of amplification of toxA, exoS, and plcH are presented in Figure 2, Figure 3, and the respective figure of plcH.

Ethical approval

The Scientific Ethical Committee of the College of Veterinary Medicine, University of Diyala, Iraq, approved this study (Approval no: Vet Medicine (235); October, 2025, W and S).

Results

Isolation and Identification of *Pseudomonas aeruginosa*

Out of 168 samples dealt with in this study, 122 (72.61%) showed growth of the bacterium, and 46(27.38%) were culture negative. Based on morphological, biochemical, and VITEK 2 analyses, ten isolates (8.19% of positive cultures) were determined to be *P. aeruginosa*. VITEK 2 system confirmed all isolates with 99% probability and 100% accuracy, proving the confirmatory identification of P. aeruginosa by this automative system.

The distribution of P. aeruginosa among various specimen types is presented in Table 3. Out of the 53 collected urine samples, 41 showed the growth of the bacterium, and 7 isolates (17.07% of positive urine cultures) were determined to be *P. aeruginosa*. This was the specimen type with the highest isolation. Concerning the 53 ear swab samples, 43 showed positive growth of the bacterium, and 3 isolates (6.97%) of positive ear cultures) were confirmed as *P. aeruginosa*. In contrast, among the 62 milk samples, no *P. aeruginosa* isolates were obtained despite bacterial growth of other organisms in 38 of the samples. Overall, *P. aeruginosa* was confirmed in 10 isolates (8.19% of all positive cultures) from the total study samples of 168.

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Table (3): The total number and proportion of P. aeruginosa isolations from various samples of cattle.

Type of samples	No. of samples	No. of positive growth	No. of *P. aeruginosa* isolates	Percentage of *P. aeruginosa* isolates (%)
Urine	53	41	7	17.07
Ear	53	43	3	6.97
Milk	62	38	0	0.00
Total	**168**	**122**	**10**	**8.19**

Note: *In Table 3, percentages are relative to positive growth samples, consistent with the original document.*

All isolates were gram negative, straight or curved bacilli, and in microscopy, were seen as either straight or curved bacilli. Colonies of *P. aeruginosa* were large, irregular, and their pigmentation smelled like grapes or fruit. In MacConkey agar, colonies that do not ferment lactose were seen as smooth, flat, and pale. Beta hemolysis (hemolytic toxins) were seen in all the isolates on blood agar.

In cetrimide agar, *P. aeruginosa* has a selective growth medium and the colonies produce yellowish green fluorescence pyoverdine, and bluish green pyocyanin. Pyocyanin is a characteristic feature that differentiates *P. aeruginosa*. Not only is this pigment a diagnostic feature, it also plays a role in gene expression, biofilm formation and is a virulence factor. **Figure 1** illustrates *P. aeruginosa* characteristic culture on cetrimide agar, with clearly developed pyocyanin pigment.

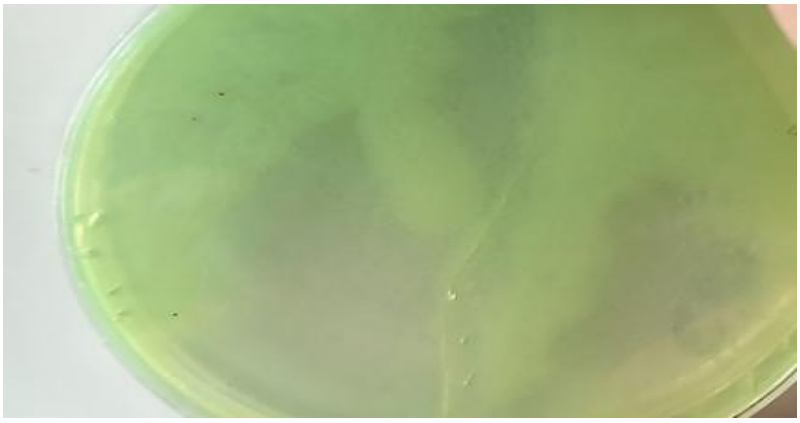

Figure 1: Cetrimide agar culture reveals *Pseudomonas aeruginosa* showing characteristic bluish-green and yellowish-green colonies.

All biochemical tests done on the ten isolates: oxidase, catalase, and gelatin hydrolysis tests and citrate utilization were positive. This shows that *P. aeruginosa* has a flexible metabolism and is

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able to sustain itself in a wide variety of environments. On the other hand, all isolates were negative for the following: indole production, urease,the methyl red test, the Voges-Proskauer test, and hydrogen sulfide (H_2S) production. These findings are consistent with the identification of *P. aeruginosa* and serve to differentiate it from other Gram-negative bacteria such as *Escherichia coli* and *Proteus* species.

Molecular Detection of Virulence Genes

A total of ten *P. aeruginosa* isolates were screened by Polymerase Chain Reaction (PCR) for three virulence genes of varying prevalence indicated by the presence of bands on 1.5% agarose gel electrophoresis for the amplified products, confirming the presence of virulence genes: *toxA*, *exoS*, *plcH*.

Detection of the *toxA* Gene

Detection of the *toxA* gene in Exotoxin A recorded the most prevalent positive isolation report. Exotoxin A is an extremely potent virulence factor in *P. aeruginosa* as an ADP-ribosyltransferase and capable of disrupting host cell protein synthesis via ADP-ribosylation of elongation factor-2 leading to the demise of the cell. We can conclude that virulence factor *toxA* in bovine isolates is significant in the development of a *P. aeruginosa* infection in cattle. Especially, the cases where the infection is located in the urinary tract or the ear, which are known as urinary tract infections and otitis, respectively.

The amplification of the *toxA* gene (745 bp) among the isolates is demonstrated in **Figure 2** displaying a 1.5% agarose gel electrophoresis result. Lane M is a marker (100-1500 bp) of the DNA ladder. Positive bands at 745 bp were seen in 9 of the 10 *P. aeruginosa* isolates and 10 lanes. Apart from lane 6 which is the only isolate that did not present a band, signifying the absence of the *toxA* gene. Bright bands indicate successful amplification of the *toxA* gene.

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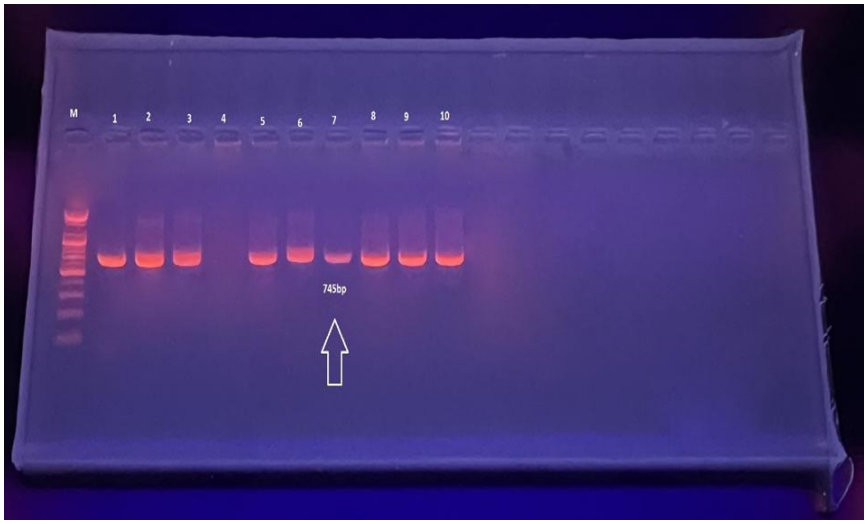

Figure 2: Amplification of the *toxA* gene for *Pseudomonas aeruginosa* isolates run on a 1.5% agarose gel electrophoresis and staining with ethidium bromide (EtBr). M: ladder marker. Lanes (1-10) are *Pseudomonas aeruginosa* isolates. *(Shows 745 bp bands in 9 out of 10 lanes)*

Detection of the *exoS* Gene

The *exoS* gene was detected in 5 of 10 isolates (50%), which means that *exoS* (exoenzyme S) is present. Exoenzyme S is a type III secretion system effector, which means it enters the target cell and disrupts the signaling and cytoskeletal network. This promotes bacterial infection and the avoidance of host immune detection. In comparison, the gene prevalence for *exoS* is lower than for the *toxA* gene, which indicates the isolation source of the isolates and may reflect strain-specific variation in the potential virulence of the isolates.

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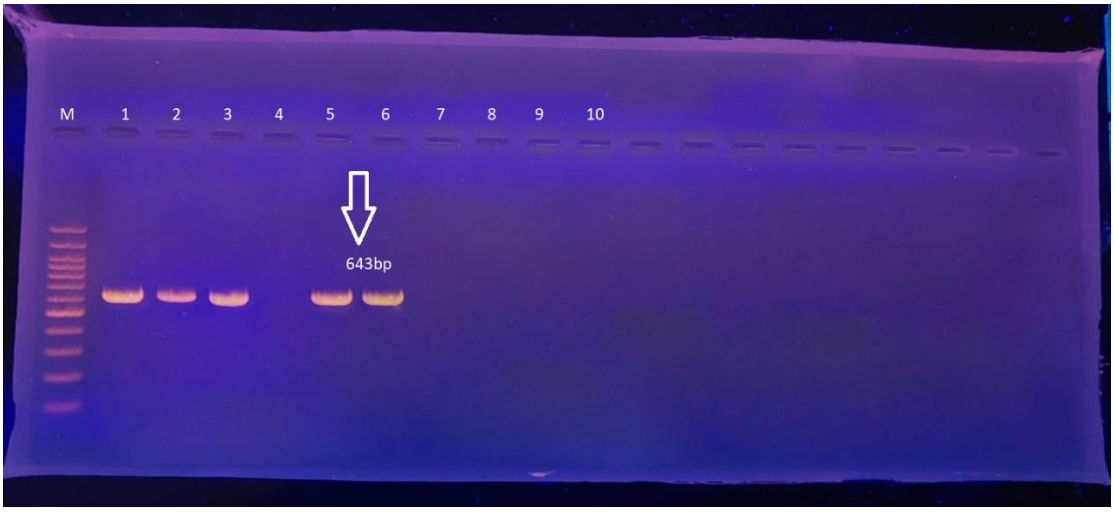

Figure 3 shows the *exoS* gene amplification product (643 bp) seen on 1.5% agarose gel electrophoresis. M is the 100-1000 bp DNA ladder marker. Positive bands for 643 bp are present in lanes 1, 3, 5, 7, and 9 for the 10 *P. aeruginosa* isolates, while the remaining lanes (2, 4, 6, 8, and 10) tested negative for the *exoS* gene.

plcH Gene Detection

The *plcH* gene which encodes for hemolytic phospholipase C, was found in 7 out of 10 isolates. Phospholipase C destroys host cell membrane phospholipids leading to tissue damage, hemolysis, and the dispersal of the bacteria. This enzyme also disrupts surfactant in the lungs which is especially pertinent for respiratory infections.

The amplification of *plcH* gene (608 bp) in 1.5% agarose gel electrophoresis showed positive bands in 7 lanes (gel not shown but referenced in the original document). The 70% prevalence rate shows *plcH* is a common virulence determinant in bovine *P. aeruginosa* isolates from Baqubah, Iraq.

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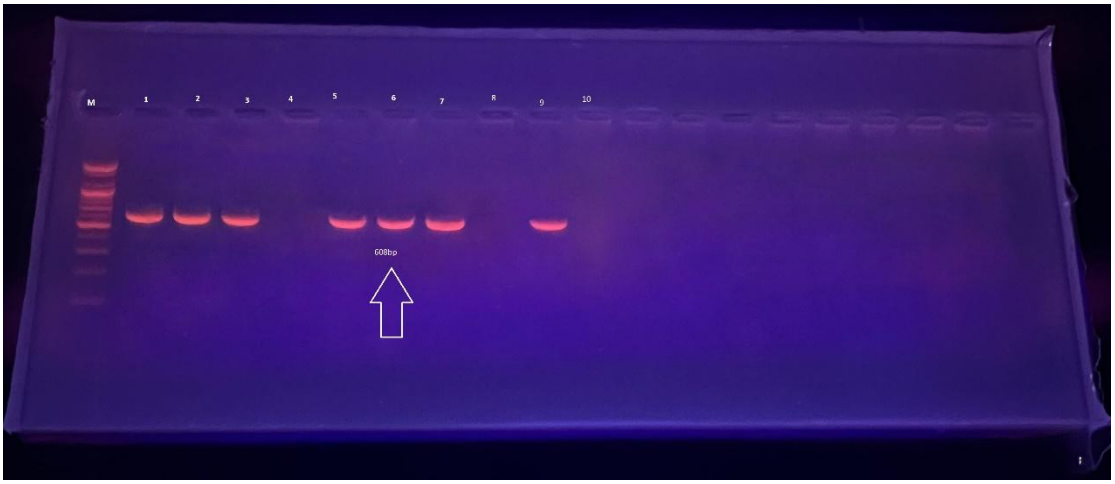

Figure 4.Amplification of *PIcH* gene of *Pseudomonas aeruginosa* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100-1000bp) bp ladder marker. Lanes(1-10) represent Pseudomonas aeruginosa isolates.

Summary of Prevalence of Virulence Genes

The prevalence of the three virulence genes in ten *P. aeruginosa* isolates were summarized in **Table 4**. The highest prevalence was observed in the *toxA* gene (90%), followed by *plcH* (70%), and then *exoS*, which had a prevalence of 50%. It was not uncommon for several virulence genes to be found in combination in a single isolate; for example, many isolates contained two or even all three genes. The presence of multiple virulence genes in an isolate may increase the pathogenic capacity of the isolate and worsen the infections in the animals.

Table (4): Summary of prevalence of virulence genes in P. aeruginosa isolates

Gene	Gene Product	Function	Positive Isolates (n=10)	Prevalence (%)
toxA	Exotoxin A	ADP-ribosylation of EF-2, inhibits protein synthesis	9	90
exoS	Exoenzyme S	Type III secretion effector, disrupts host cell signaling	5	50
plcH	Hemolytic phospholipase C	Degrades phospholipids, causes hemolysis and tissue destruction	7	70

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Discussion

Isolation Rate and Specimen Distribution

The current study was able to isolate *P. aeruginosa* from 8.19% of positive cultures from bovine clinical specimens in Baqubah, Iraq. *P. aeruginosa* was most frequently isolated from urine samples, then from ear swabs, and no isolates were recovered from milk samples (**Table 3**). These findings aid the understanding of the distribution of *P. aeruginosa* infections in cattle in this area.

The findings from this study echo the previous literature which state that *P. aeruginosa* can be isolated in high numbers in urine samples, as it is a known uropathogen. *P. aeruginosa* is well known to be involved in Urinary Tract Infections (UTIs)/cystitis as it is able to stick to uroepithelial cells, form a protective biofilm and can withstand the immune response (Jasim *et al.*, 2024) i.e. the bacteria can survive even if the body is trying to get rid of it. Uropathogenesis is one of the reasons for the isolation of *P. aeruginosa* from UTIs in Iraqi patients (Abed *et al.*, 2024). *P. aeruginosa* has uropathogenic virulence factors which enable it to cause a chronic infection in the urinary tract (Milivojevic *et al.*, 2018). These virulence factors include adhesins (those that help the bacteria stick to cells), toxins (toxic substances that can injure or kill cells) and biofilms (a protective layer that allows bacteria to survive in harsh conditions) which all help to establish and maintain infections.

The isolation of *P. aeruginosa* from ear swabs as 6.97% of positive cultures (**Table 3**) illustrates the association of this bacterium with otitis externa in animals. Al-Tememe and Abbas (2022) isolated *P. aeruginosa* from ear swabs of infected ruminants in Basrah, Iraq, which is in agreement with the present study. Colonization of the ear canal by *P. aeruginosa* is associated with its production of adhesins and biofilm formation which protects the bacteria from host immune response and antibiotics (Milivojevic *et al.*, 2018). Isolation of *P. aeruginosa* from milk samples in this study in contrast to other studies conducted in Iraq. Jasim *et al.* (2024) reported *P. aeruginosa* in 60% of bovine milk samples from Basra province. Similarly, Saeed *et al.* (2023) reported 11 positive isolates from 70 raw cow milk samples collected in Diwaniya and Al-Najaf. Nevertheless, the recovery rate of *P. aeruginosa* from milk varies with the hygiene practiced during milking and disinfection of containers (Banda *et al.*, 2019). About 80% of dairy farmers do not disinfect milk containers, which may affect the contamination pattern (Banda *et al.*, 2020). A study conducted in China reported *P. aeruginosa* in raw milk samples, and the variation in the isolates was attributed to the farm management (Raw Milk Study, 2025).

Prevalence of Virulence Genes and Pathogenesis

It is remarkable that the *toxA* gene is highly prevalent (90%, **Table 4** and **Figure 2**) among the isolated strains of *P. aeruginosa*. Exotoxin A is one of the most powerful provided virulence

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factors of *P. aeruginosa* (Al-Muthanna study, 2025). The high number of bull isolates suggests that exotoxin A is significant in the pathogenesis of bovine *P. aeruginosa* infections. The study of bovine mastitis due to *P. aeruginosa*, *toxA* was one of the most frequently found virulence factors that contributed to intensifying the intramammary infection (Bovine mastitis study, 2024). The presence of the *plcH* gene in 70% of isolates (**Table 4**) suggests that phospholipase C is also an important virulence factor in bovine *P. aeruginosa* strains. Phospholipase C causes the breakdown of phospholipids in the membranes of host cells, leading to tissue damage and the further dissemination of bacteria (Özsoy *et al.*, 2024; Mohammed and Alshammary 2025). This is consistent with the studies on *P. aeruginosa* isolates from different clinical sources, in which *plcH* was frequently identified (Al-Muthanna study, 2025).

The *exoS* gene is found in 50% of isolates (**Table 4** and **Figure 3**) and encodes exoenzyme S, an effector of the type 3 secretion system that interferes with host cell signaling and the cytoskeletal system (Özsoy *et al.*, 2024). Compared to the other genes, *toxA* and *plcH*, the moderate prevalence of *exoS* may be related to the variation in the strain's pathways in the virulence potential. It has been suggested that the variation in the geographical distribution of virulence factors for *Pseudomonas aeruginosa* isolates is related to the ability of these bacteria to adapt to different environmental situations during their infections (Study in Iraqi clinical isolates, 2026). Zghair and Mathree (2025) found *exoS* in their Iraqi isolates in 40-60% and their findings depend on the isolation site, which is consistent with our data. Some of the isolates showed the presence of multiple virulence genes, such as having both *toxA* and *plcH*. This single or consortium virulence factor-containing strain indicates the presence of a significant virulence potential. The presence of *toxA*, *plcH*, and *exoS* may further increase the virulence of the strains by the coordinated action of inhibition of host protein synthesis, destruction of host cell membranes, and the disruption of cellular signaling pathways.

Phenotypic Characteristics

Figure 1 illustrates the pigmentation characteristics of *P. aeruginosa* on cetrimide agar, which is the *Pseudomonas* species attraction (Kamil *et al.*, 2023). Pyocyanin pigment, which is produced by the species, functions as a virulence factor via biofilm production and gene expression (Rigan *et al.*, 2020). Reports by Jawhar and Hasan (2022) state that the *P. aeruginosa* isolates from the meat samples collected from the city of Mosul, Iraq, showed pigmentation on cetrimide agar, which aided in the identifications of the samples.

In this study, we conducted an analysis of the isolates, and they showed a compatible biochemical profile which included characteristics such as, that which included oxidase, catalase, urease, and citrate to be positive while the H_2S, VP, and Indole tests to be negative which aligned with the findings of Alonso *et al.* (2020) to be true in regard to the spaces left as the characteristics of *P. aeruginosa*. Some biochemical traits demonstrate the metabolic adaptability of *P. aeruginosa* and its opportunistic ecological success as a pathogen.

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Comparison with Regional and International Studies

Finding similarities in the context of *P. aeruginosa* virulence factors research conducted for the given region and the results from this study. In Iraq, research conducted by Taha (2025) showed positive virulence factor research from *P. aeruginosa* isolates collected from trout farms across the country. In addition, Zghair and Mathree (2025) reported the same prevalence of *exoS* as well as type III secretion system gene variants in human and chicken isolates from the Wasit province. The study's use of the VITEK 2 system for confirmatory identification is consistent with Iraqi diagnostic laboratory standards. The reliability of the VITEK 2 system was confirmed by Abed *et al.* (2024) and Jasim *et al.* (2024) when accurate identification of *P. aeruginosa* from laboratory clinical samples was performed.

Conclusions

Isolation and identification of *P. aeruginosa* from bovine clinical specimens in Baqubah, Iraq with the greatest isolation rate from the urine sample (**Table 3**). VITEK 2 has proven to be one of the most accurate and reliable methods for the confirmation of *P. aeruginosa* identification. The characteristic pigmentation of *P. aeruginosa* on cetrimide agar (**Figure 1**) is a good phenotypic marker. Molecular analysis by PCR, with specific primers (**Table 1**) and optimized thermal cycling (**Table 2**), showed virulence-associated gene prevalence, with 90% of the isolates (**Figure 2**, **Table 4**) tested positive for *toxA*, 70% for *plcH*, and 50% for *exoS* (**Figure 3**). These virulence genes demonstrated bovine *P. aeruginosa* isolates' pathogenic potential. Virulence determinants in *P. aeruginosa* were demonstrated that PCR is a rapid, specific, and efficient method. These results warrant the surveillance on *P. aeruginosa* infections in livestock and biosecurity measures to control the spread of *P. aeruginosa* from animals to humans.

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Conflict of Interest:

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Authors Contributions:

The author was solely responsible for the conception, design, sample collection, laboratory experiments, data analysis, and manuscript preparation of this study.

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