Tikrit Journal of Veterinary Sciences (2026) 5(1): 36-43
DOI : 10.25130/tjvs.5.1.5

IRAQI Academic Scientific Journals

TJVS Tikrit Journal of Veterinary Sciences

Tikrit Journal of Veterinary Science

Histopathological changes of the small intestine using a standard dose of copper sulfate in rats (feed supplement dose)

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ARTICLE INFO.

Article history:
-Received: 8/10/2025
-Received In Revised Form:21/11/2026
-Accepted: 23/11/2025
-Available online: 30/6/2026

Keywords:
Pathogenicity, PCR, Pneumolysin, pneumonia, Streptococcus, Virulence factors.

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ABSTRACT

Background & aim: The present study aimed to detect the pneumolysin (*ply*) virulence gene in *Streptococcus pneumoniae* isolates obtained from human clinical samples. **Materials and Methods**: A total of 50 isolates were collected from patients suffering from respiratory tract infections between November and February at Tikrit Teaching Hospital and Al-Tawfiq Private Hospital, Iraq. Blood agar was used for the cultivation of isolates, and identification was performed using the VITEK 2 system. Molecular detection of the *ply* gene was carried out by polymerase chain reaction (PCR). **Results:** The results of the present study showed that 35 out of 50 isolates (70%) were positive for the *ply* gene, indicating a high prevalence of this virulence factor among local pneumococcal strains. **Conclusions**: These findings emphasize the importance of molecular methods for the rapid diagnosis and surveillance of virulent *Streptococcus pneumoniae* strains in clinical settings.

Introduction

Streptococcus pneumoniae (S. pneumoniae) is facultative anaerobic, Gram-positive bacteria responsible to severe infections such as otitis media, meningitis, and pneumonia, also is main cause of mortality and morbidity worldwide, particular in immunocompromised individuals and children **[1]**. The pathogenicity of *S. pneumoniae* is determined by virulence factors, including pneumolysin (Ply), a pore-forming cytotoxin that plays a necessary role in host tissue damage and evasion from immunity system **[2]**. The identification of molecular virulence genes provides powerful method to assess virulence potential of bacterial isolates and aids in epidemiologically surveillance and vaccine development. Some of studies have elucidated the contribution of pneumolysin in pathogenesis of pneumococcal infection. Ply has been implicated in damage epithelial cells, activates complement pathways, and induced inflammatory cytokines such as TNF-α and IL-1β **[3]**. According to **[4]**, Ply-deficient mutants exhibit significant reduce virulence in murine models. Furthermore, **[5]** showed that ply is among the most conserved genes across pneumococcal strain, making it an ideal marker for molecular diagnosis. Recent advances in PCR and sequence technique have enhanced our ability to rapid identify such virulence association genes in clinically isolates **[6]**. *Streptococcus pneumoniae* is Gram-positive bacteria belong to the cocci group. It is characterizing by its spherical shape and often occurs in pairs (diplococci). It is a facultative anaerobe. This bacterium is important causes of bacterial infections in humans, especial in immunocompromised groups such as elderly and children. Diseases caused by it include bacteremia, sinusitis, otitis media, meningitis, and pneumonia **[1]**. *S. pneumoniae* possesses virulence factors that enable it to colony the host, evade the immunity system, and cause cells damage. One of the most prominent of these factors is pneumolysin, a toxin produces by *S. pneumoniae*, which is one of the main factors enable the bacteria to cause tissue damage and overcome the host defenses **[2].** Pneumolysin plays a necessary role in development of many diseases cause by S. pneumoniae, especial pneumonia and meningitis, where it contributes to severe inflammation and damage to lung or brain tissues **[7]**. These bacterial causes a number of diseases, the most important of which are ear infections, meningitis and pneumonia. The infections are usually transmitted during sneezing or coughing from an infected person and also spread by touching contamination surfaces and then touch nose or mouth. The bacteria are found in upper throat of some healthy people and can enter lungs during occur disturbances in immune system. These bacteria can cause inflammatory infections in a person in conditions, such as respiratory tract infections or weakened immune system **[8].** Infection with these bacteria can be prevented and treatment in several methods by antibiotics as first line of treatment, and supportive therapies such as providing fluids and oxygen. Also can used vaccination for S. pneumoniae strains such as the pneumococcal vaccine. In addition, also important hand washing and minimizing contact with infected people **[1]**. The methods for isolate *S. pneumoniae* involves taking a samples from a sterile place in the body, such as sputum, middle ear fluid, joint fluid, cerebrospinal fluid, or blood and then perform a Gram stain, and culture on blood agar for identify alpha-lytic isolates. The isolation is certain by a sensitivity test of optochin and a bile salt solubility test **[9].** The Aims of the present study:

1. To isolate S. pneumoniae from human clinical samples.
2. To diagnose S. pneumoniae using the VITEK 2 system and polymerase chain reaction (PCR) to confirm bacterial identity and detect species-specific genes.
3. To use PCR to detect the virulence factor (pneumolysin) of *Streptococcus pneumoniae* by targeting genes associated with pathogenicity.
4. To incubate the isolates in laboratory animals and determine any changes in virulence when the bacteria are transferred from humans to experimental animals.

Materials and Methods

Sample Collection

Fifty blood and sputum samples were collected from patients of both sexes aged between 15 and 75 years old, showing clinical signs of lung disease (pneumonia). The samples were obtained from government and private hospitals in the city of Tikrit, represented by Tikrit Teaching Hospital and Al-Tawfiq Private Hospital, during the period from November 2024 to February 2025. The samples were transported immediately after collection to the central laboratories/University of Tikrit, under sterile conditions to preserve sample integrity and prevent accidental contamination.

Each sample was treated individually, whereby part of the sample was cultured on blood agar and the dishes were incubated at 37°C for 24 hours

under aerobic conditions for the initial detection of possible colonies of *S. pneumoniae* bacteria. After the appearance of characteristic colonies, the number of positive samples (35 samples) and the number of negative samples (15 samples) were determined. A small loop or swab from each sample was transferred to a selective medium specifically designed to isolate *Streptococcus spp* bacteria. This medium was prepared to contain the antibiotic gentamicin to inhibit the growth of unwanted bacteria, especially *Staphylococcus spp* **[10].**

Diagnosis of bacterial isolation

After colonies grew on Blood Agar medium, two tests were performed to confirm the bacterial identity of S. pneumoniae as follows:

First test – Automated diagnosis using the VITEK 2 Compact device:

The VITEK 2 Compact device (bioMérieux, France) was used to automatically identify the bacteria based on the chemical and biological characteristics of the colonies. The device analyses the reaction patterns on Gram-positive organism cards (GP cards) and compares the results with a standard database, allowing the bacterial species to be identified with high accuracy **[11].**

Second test – genetic confirmation using polymerase chain reaction (PCR) technology:

PCR was performed to confirm the genetic identity of *Streptococcus pneumoniae* by detecting the species-specific Pneumolysin (ply) gene, which is one of the most important diagnostic genes for this species. DNA was extracted from pure colonies, and the reaction was performed using specific primers, with positive and negative controls included in each run to ensure the reliability of the results **[11].**

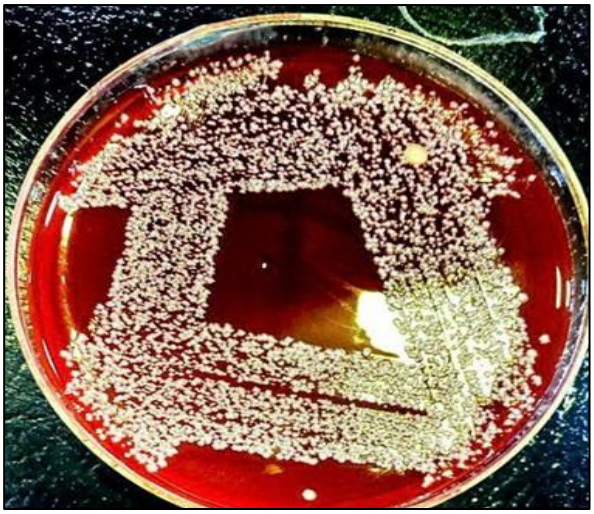
Fifty clinical specimens were collected from patients presenting respiratory infections in Tikrit city hospitals. Blood agar was used for bacterial isolation, and colonies exhibiting alpha-hemolysis were subjected to biochemical identification via the VITEK 2 Compact system. Genomic DNA was extracted using a commercial kit, and the presence of the pneumolysin gene (ply) was detected by PCR using specific primers (Forward: CTGAAGTTAAAGGCTGTGGCTTA; Reverse: GACTTAACCCAACATCTCACGAC). PCR amplification was performed under standard conditions, and amplified products were visualized on 1.5% agarose gel electrophoresis stained with ethidium bromide.

Results

Out of 50 *Streptococcus pneumoniae* isolates, 35 (70%) were found positive for the pneumolysin gene. The PCR products produced an amplicon of 348 bp, consistent with the expected size. The distribution of the gene was higher among isolates from patients with severe pneumonia. Table 1 summarizes the prevalence of the ply gene among the studied isolates.

Characteristic Bactria on blood agar

All S. pneumonia isolates were highly sensitive to heat and desiccation. They were isolated and cultured only on blood agar, and their optimal growth temperature was 35°C. These isolates showed poor growth under aerobic conditions, while their best growth was in the presence of 2–5% carbon dioxide, which is usually provided using a candle jar. All young colonies (18–24 hours old) were convex in shape at the beginning of their growth. Twenty isolates formed small, gray, moist colonies with a diameter of up to 1 mm, while only four isolates were mucoid and large, with a diameter ranging from 2–4 mm. All isolates showed alpha-type hemolysis as a phenotypic characteristic, manifested as a green halo around each colony. After 24 hours of incubation, the colonies began to take on a low shape in the center and a high shape at the edges. When incubation continued for more than 24 hours, the colonies began to disappear from the culture dishes (fig 1).



Tikrit Journal of Veterinary Sciences (2026) 5(1): 36-43
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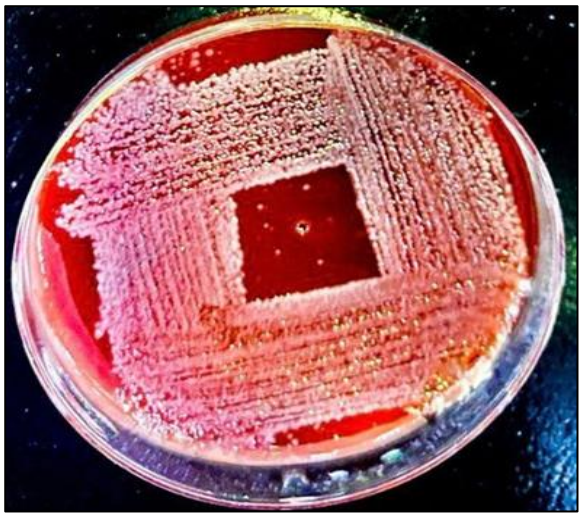

Figure (1): Growth of Streptococcus pneumoniae on blood agar after incubation at 37°C for 24 h, showing gray colonies surrounded by a zone of alpha-hemolysis (a-hemolysis).

Microscopic characteristics

All isolates of S. pneumoniae were Gram-positive, double cocci, arranged in pairs, and not observed in chains, appearing lancet-shaped. They were surrounded by a capsule, which appeared as a transparent zone surrounding the bacteria on the center of the blood agar.

Diagnosis of S. pneumonia bacteria using the VITEK device

Three samples were taken within the scope of the current study for the purpose of confirming the diagnosis of bacteria within the scope of the current study, and the diagnosis was performed using the VITEK device. The first sample was from a 45-year-old male patient suffering from clinical symptoms consistent with pneumonia. The second sample was from a 48-year-old man with similar symptoms of pneumonia. The third sample was taken from a 55-year-old man suspected of having pneumonia, as well as clinical symptoms and signs indicating pneumonia. The results were as shown in the VITEK report, where the test period was 5.82 hours and the diagnosis rate was 91% for the first sample, indicating that the person had a high rate of infection with this bacterium in the current study. The diagnosis rate for the second sample was 87% with a period of 5.78 hours. The diagnosis rate for the third sample was 87%, which was equal to the diagnosis rate for the second sample, and the diagnosis period was 5.03 hours. This indicates that the three samples had a high rate of infection with S. pneumonia bacteria. Table (1).

Table (1): Biochemical identification results of Streptococcus pneumoniae obtained using the VITEK 2 system, showing a probability of identification of 87% (Analysis time: 5.03 h; Status: Final; Bionumber: 061133334201471).

	Test	Result		Test	Result		Test	Result
2	AMY	-	4	PIPLC	-	5	dXYL	-
8	ADH1	-	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-
16	BGAR	+	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	+	24	BGURr	-
25	AGAL	+	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-
31	URE	+	32	POLYB	+	37	dGAL	-
38	dRIB	+	39	ILATk	-	42	LAC	+
44	NAG	+	45	dMAL	+	46	BACI	-
47	NOVO	-	50	NC6.5	-	52	dMAN	+
53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	+
60	SAC	+	62	dTRE	+	63	ADH2s	+
64	OPTO	+						

The Result of The Diagnosis of S. pneumoniae genes using the PCR molecular method

The results of the molecular test, as shown in Table (2), confirmed the diagnosis of *S. pneumoniae* bacteria according to the nucleotide sequence F CTGAAGTTAAAGGCTGTGGCTTA 23 and the nucleotide sequence R GACTTAACCCAACATCTCACGAC 23 specifics to it, as well as the diagnosis of the virulence factor hemolysin according to the nucleotide sequence Play F AAATCACGGCTCACAGCATG 20 and the

nucleotide sequence shown in Table (2) Play R TAGGAAATCGGCAAGCCTGG 20.

Table (2): Diagnosis of S. pneumonia bacteria and genes under study using PCR technology.

	Primer name	Organism	t
F CTGAAGTTAAAGGCTGTGGCTTA 23 R GACTTAACCCAACATCTCACGAC 23 Annealing 58 product 498	Diagnostic Primer	*S.pneumonia*	**1**
Ply F AAATCACGGCTCACAGCATG 20 Ply R TAGGAAATCGGCAAGCCTGG 20 Annealing 59 product 538	Pneumolysin	*S.pneumonia*	**2**

Discussion

The high detection rate of the ply gene supports its critical role as a virulence determinant. Similar findings were observed by [12], who reported 68% positivity among clinical isolates. S. pneumoniae pathogenic potential is highlighted by its capacity to lyse host cells and induce inflammatory reactions, evolutionary pressure to preserve its function is indicated by the genetic conservation of ply across strains. These findings are consistent with earlier research the significance of pneumolysin as a target for vaccines and its function in invasive pneumococcal infections [13]. Gram-positive, lancet-shaped diplococci that were capsulated and exhibited alpha-hemolysis on blood agar were discovered during the isolates classical microbiological characterization these results closely match the known S. pneumoniae [14]. These bacteria is easily distinguished from other type of streptococci, which usually occur in longer chains, by its diplococcal organization and distinctive lanceolate morphology [15]. Capsule is the primary virulence factor of S. pneumoniae, giving antiphagocytic protection and elucidating its association with invasive illnesses like meningitis and pneumonia [13-16]. The identification is another supported by alpha-hemolysis, which appears as a zone surrounding bacterial colonies and represents partial red blood cell lysis caused by pneumococcal metabolites, specifically pneumolysin. [17]. In result, significant presumptive evidence for the diagnosis of S. pneumoniae is provided by the combined characteristics of diplococcal shape, capsulation, alpha-hemolysis, and Gram-positive staining. These traditional results serve as the crucial first step before molecular confirmation via PCR that targets virulence genes like lytA or ply, guaranteeing a precise and thorough diagnosis. The three clinical isolates in the current investigation had high probability values of 91%, 87%, and 87% for the diagnosis of Streptococcus pneumoniae utilizing the VITEK automated system, with analysis times ranging from 5.03 to 5.82 hours. These findings suggest a high probability of S. pneumoniae infection in the patients under investigation, all of whom had pneumonia-like clinical symptoms. The obtained biochemical pattern, particularly the optochin sensitivity (OPTO = +), further supports the diagnosis, as this characteristic remains one of the most reliable phenotypic markers for S. pneumoniae differentiation [18]. VITEK system offers rapid and perfect identification for bacteria, previous studies showed possible misidentification within the Streptococcus mitis group due to overlapping biochemical profiles [19]. confirmatory tests like bile solubility or molecular amplification targeting the lytA gene is recommended to achieve definitive identification [20] The obtained probability values (>85%) fall within the accepted diagnostic range reported by recent evaluations of the VITEK 2 Compact system for pneumococcal identification [21-22]. Clinically, the consistent diagnostic of S. pneumoniae in three symptomatic patients strongly suggests its cause's role in pneumonia within the study population. However, combining rapid automated system with confirmatory molecular assays remains essential to ensure diagnostic accuracy and to guide appropriate antimicrobial therapy. Molecular identification of Streptococcus pneumoniae using PCR revealed amplification of the expected fragments for both the diagnostic gene (498 bp, annealing at 58 °C) and the pneumolysin (ply) virulence gene (538 bp, annealing at 59 °C). The successful amplification of these two targets confirms the presence of S. pneumoniae DNA in the examined isolates. The ply gene encodes pneumolysin, a cholesterol-dependent cytolysin that contributes to tissue damage, complement activation, and inflammatory reaction, which are points features in the pathogenesis of S pneumonia [19]. The diagnostic of virulence-associated genes strongly supports the phenotypic findings obtained by the VITEK system and confirms the isolates as S. pneumoniae. These molecular findings provide additional evidence of the virulent potential of the

recovered strains, highlighting their clinical relevance in respiratory infections. Nevertheless, molecular assays targeting ply alone may occasionally yield false-positive reactions due to sequence similarity among members of the Streptococcus mitis group [22]. Therefore, combining ply-based PCR with highly specific markers such as lytA, piaB, or SP2020 is recommended to enhance accuracy and minimize misidentification [19-20]. Methodologically speaking, the PCR parameters used in this investigation, such as primer specificity and annealing temperatures, are in line with previously approved procedures. To guarantee dependability and rule out inhibition or contamination, it is still necessary to employ suitable positive and negative controls in addition to an internal amplification control. Sequencing representative PCR products or lytA-based real-time PCR assays can be employed as complementary methods for final identification [23]. In summary, the molecular finding in a study confirmed the presence of S. pneumoniae in the samples and indicated the expression of the pneumolysin virulence gene, which is directly associated with the pathogen's invasive potential. Combining molecular and biochemical data improve the pathogenic mechanisms of pneumococcal infections and offers a strong basis for precise diagnosis.

Conclusion

The pneumolysin gene molecular identification offers a quick and accurate pathway to estimate Streptococcus pneumoniae potential for virulence. The necessity of that includes molecular diagnostics into normal bacteria test shown by the substantial relation between the presence of ply and clinical severity. The genetic variety of virulence genes among various serotypes and its consequences for vaccine design should be investigated in more detail.

Acknowledgement

The authors are grateful to the Tikrit University /College of Veterinary Medicine for all the facilities to achieve this study.

Declaration of interests

The authors declare no competing interests.

Funding Sources

This research received no financial support or specific grant from any funding agency in the commercial, public, or not-for-profit sectors.

Publication consent

Accepted for publication in Tikrit Journal of Veterinary Sciences (TJVS), Vol. 1, No. 1 (2026), article entitled "Molecular Identification of Virulence Genes (ply) in Streptococcus pneumoniae Isolated from Respiratory Tract", Acceptance Letter Ref. No. 117, dated 23 Nov 2025.

Data and material availability

All data analyzed and generated in this study are included in this published research.

Author contribution

All authors participated in the study design and conception. Data analysis, data collection, performance of the results, and assent to the final version.

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Tikrit Journal of Veterinary Sciences (2026) 5(1): 36-43
DOI : 10.25130/tjvs.5.1.5

التغيرات النسيجية المرضية في الأمعاء الدقيقة لدى الجرذان عند استخدام جرعة قياسية من كبريتات النحاس (جرعة مكمل غذائي)

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الملخص

الخلفية والهدف: هدفت الدراسة الحالية إلى التحري عن جين الضراوة النيمولايسين (Pneumolysin, ply) في عزلات بكتيريا المكورات الرئوية (Streptococcus pneumoniae) المعزولة من العينات السريرية البشرية.
المواد وطرائق العمل: جُمعت خمسون عزلة بكتيرية من مرضى مصابين بالتهابات الجهاز التنفسي خلال المدة من تشرين الثاني إلى شباط من مستشفى تكريت التعليمي ومستشفى التوفيق الأهلي في العراق. زُرعت العزلات على وسط آغار الدم، وشُخِّصت باستخدام نظام VITEK 2. كما أُجري الكشف الجزيئي عن جين (ply) باستعمال تقنية تفاعل البوليميراز المتسلسل (PCR).
النتائج: بينت نتائج الدراسة أن 35 عزلة من أصل 50 عزلة (70%) كانت موجبة لجين(ply) ، مما يشير إلى الانتشار المرتفع لهذا عامل الضراوة بين السلالات المحلية لبكتيريا المكورات الرئوية.
الاستنتاجات: تؤكد هذه النتائج أهمية التقنيات الجزيئية في التشخيص السريع ورصد السلالات الضارية من بكتيريا المكورات الرئوية، مما يسهم في تحسين برامج المراقبة الوبائية وتعزيز استراتيجيات الوقاية والسيطرة على العدوى في البيئات السريرية.

الكلمات المفتاحية: المكورات الرئوية؛ عوامل الضراوة؛ نيمولايسين؛ تفاعل البلمرة المتسلسل؛ الامراضية.