



ISSN: 0067-2904

Isolation and Identification of Novel *Streptococcus pluranimalium* Isolated from Children with Upper Respiratory Infections in Mosul Hospitals, Iraq

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Received: 2/9/2021

Accepted: 21/11/2021

Published: 30/9/2022

Abstract

Streptococcus pluranimalium was first isolated in 1999. Recently, several case reports have been published that have revealed that *S. pluranimalium* can infect humans as well. The pathogenicity and pathophysiology of this pathogen is poorly studied and its characteristics are not well known. In this study, *S. pluranimalium* was first isolated and then identified from infants and children who suffered from upper respiratory infections. 90 samples were collected from nasopharyngeal cavity. Among them, 83 *Streptococcus* spp. isolates were identified. 3 out of which were biochemically and molecularly identified as *S. pluranimalium*. 16S rRNA sequencing based identification revealed that all isolates showed a very close identity match (92%) with subjected *S. pluranimalium* when blasted with gene bank database. Furthermore, the antibiotic susceptibility of *S. pluranimalium* towards ten antibiotics was conducted using Kirby-Bauer disk diffusion test. The results revealed that *S. pluranimalium* was 100% susceptible to penicillin, levofloxacin, and ofloxacin. However, other antibiotics showed diverse effects on *S. pluranimalium*.

Keywords: *Streptococcus pluranimalium*, 16S rRNA sequencing, antibiotic susceptibility.

عزل وتشخيص *Streptococcus pluranimalium* المعزولة لأول مرة من الاطفال المصابين باخماج الجهاز التنفسي العلوي في مدينة الموصل، العراق

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الخلاصة

تعد جرثومة *S. pluranimalium* من الانواع المعزولة من الحيوانات الداجنة والتي تربي في البيوت في عام 1999. تم نشر بعض حالات مرضية من الانسان اوضحت ان *S. pluranimalium* بإمكانه ان يصيب الانسان ايضا . الامراضية وسلوك هذا الممرض غير معروف الى حد الان. في هذه الدراسة، عزلت *S. pluranimalium* لأول مرة في العراق من الاطفال المصابين باخماج الجهاز التنفسي العلوي. جمعت 90 عينة مرضية وشخصت 83 منها تشخيصا مظهريا وكموحيويا على انها *Streptococcus* spp. 3 من هذه العزلات شخصت انها *S. pluranimalium* باستخدام الاختبارات البايوكيميائية والجزيئية. بينت الدراسة ان تطابق العزلات جزيئيا بنسبة 92% مع العزلات القياسية. اوضحت الدراسة ان حساسية الجرثومة

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للمضادات الحياتية كانت متفاوتة بحيث ان نسبة المقاومة كانت 100% تجاه المضادات
 ofloxacin و levofloxacin, penicillin . بينما كانت الحساسية تجاه المضادات الحياتية الاخرى متفاوتة.

Introduction:

Streptococcus pluranimalium is a new member of *Streptococcus* spp. genus which was identified by Devriese *et al.*, in 1999 [1]. The species term 'pluranimalium' is basically named as the pathogen comes from many animals ('pluris' means many and 'animalium' means 'animals'). *S. pluranimalium* was first isolated from genital tract and tonsils of cattle, cats and goats, and respiratory tract of canary birds [1]. It mainly causes primary infections in bovine and avian species. It has been reported that *S. pluranimalium* is linked to many animals infections such as septicemia in chicken [2], subclinical mastitis in cows [1] and purulent meningo-ventriculitis in calves [3]. It is linked with abortion, still birth and vaginitis of bovine [4, 5]. Recently, *S. pluranimalium* has first time been isolated from human specimens as well [6-8].

The genus *Streptococcus* is gram positive microbe. Some species normally live in human skin, mucosal membranes, respiratory and gastrointestinal tracts. Many species that belong to genus *Streptococcus* are identified as human pathogens. Since *S. pluranimalium* was first identified as human infectious agent that was reported as a transmitted pathogen from animals to human, known as zoonosis [9]. Although the literature has described this microbe as opportunistic pathogen, it is still unclear whether the microbe is the main cause of other human infections and pathogenicity, and its pathophysiological characteristics are still unknown [8].

Previous trials to isolate and characterize *S. pluranimalium* were established using mass spectrometry, 16S rRNA sequencing and phylogenetic relationship studies [2, 10, 11]. However, the full characterization establishment of *S. pluranimalium* was done by Pan *et al.*, using whole genome sequencing and phylogenetic analysis [12]. In this study, *S. pluranimalium* was first isolated from children with upper respiratory infections. Biochemical tests, 16S rRNA sequencing and antibiotic resistance test was done for the pathogen.

Materials and Methods:

Samples Collection and Bacteria Isolation:

A total of 90 nasopharyngeal samples were collected from infants and children, aged between 6 days to 5 years old who had visited Ibn-Alatheer, Al-Khansah and Ibn-Seenah University Hospitals for children. Children showed some respiratory problems including pneumonia, pharyngitis and bronchitis. The samples were taken from children's nasopharynx using cotton transport swab media to keep them fresh. The samples were directly inoculated in rich media including blood agar (5% sheep blood) and brain heart infusion broth (BHI). The samples were then incubated at 37°C for 18-24 hours. After that, the cultures were examined for morphological characteristics and blood hemolysis. Optochin susceptibility of isolated colonies was tested using Mueller-Hinton agar plates. Gram stain of isolates was also examined. The isolates were then preserved in BHI broth supplemented with 15% glycerol and were then stored at -80°C until use.

Biochemical Test:

Biochemical tests were carried out for discrete colonies that showed Gram positive cocci, transparent and greenish pinpoint colonies with α -hemolysis and optochin susceptible isolates. The biochemical tests included in this study were catalase test, oxidase test, Voges proskauer test, arginine utilization test using arginine hydrolase agar, sugar fermentation test and esculin hydrolysis test using bile esculin agar [13, 14].

16S rRNA Based Detection of *S. pluranimalium*:

The positive streptococcal isolates, identified by morphological and biochemical characteristics, were subjected to molecular identification of 16S rRNA gene sequencing. For that, DNA isolation and purification of bacterial samples were done by using DNA extraction kit provided by Geneaid Biotech company (Taiwan). The purified genome DNA were then

run on agarose electrophoresis to confirm its presence and purity. The DNA samples were then stored at -20°C until use. For 16S rRNA gene amplification, conventional PCR was used to amplify the target gene using the primers (Forward, 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse, 5'-ACGGCTACCTTGTACGACTT-3'). Thermal cycler condition used included 1 cycle of initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min and final extension step of 72°C for 10 min [15]. The PCR amplicons were then run on agarose gel electrophoresis and were later visualized under UV light. The amplicons were then cut from agarose gel and purified using DNA extraction kit (Geneaid Biotech., Taiwan). The purified amplicons were sent for sequencing and the resulting data was then blasted against gene bank database using BLATn analysis provided from National Centre for Biotechnology Information (NCBI).

Antibiotic Susceptibility Test:

Antibiotics susceptibility test was carried out using Kirby-Bauer disk diffusion test. The results followed the guidelines of Clinical and Laboratory Standards Institute (CLSI) [16]. A single colony of *S. pluranimalium* was inoculated in 5 ml of brain heart infusion broth and incubated for 18-24 h at 37°C . The overnight culture was then centrifuged at 5000 rpm for 10 min. The pellet was then resuspended in 5ml of normal saline and standardized against MacFarland suspension at the concentration of 1.5×10^8 cfu/ml. The bacterial cell suspension was then spread on freshly prepared Mueller-Hinton agar plates using sterile cotton swabs. The plates were left at room temperature to dry. The antibiotic discs were then placed on the surface of inoculated plates that were incubated at 37°C for 18-24 hours. The antibiotics used in this study were penicillin (10 $\mu\text{g}/\text{disc}$), ofloxacin (5 $\mu\text{g}/\text{disc}$), levofloxacin (5 $\mu\text{g}/\text{disc}$), cefotaxime (30 $\mu\text{g}/\text{disc}$), erythromycin (10 $\mu\text{g}/\text{disc}$), chloramphenicol (10 $\mu\text{g}/\text{disc}$), ceftriaxone (30 $\mu\text{g}/\text{disc}$), azithromycin (15 $\mu\text{g}/\text{disc}$), clindamycin (10 $\mu\text{g}/\text{disc}$) and vancomycin (30 $\mu\text{g}/\text{disc}$). The antibiotic discs were provided by Bioanalyase Inc., Turkey. The results were interpreted as sensitive, intermediate and resistant according to the standard guidelines of CLSI [16].

Ethical Statement:

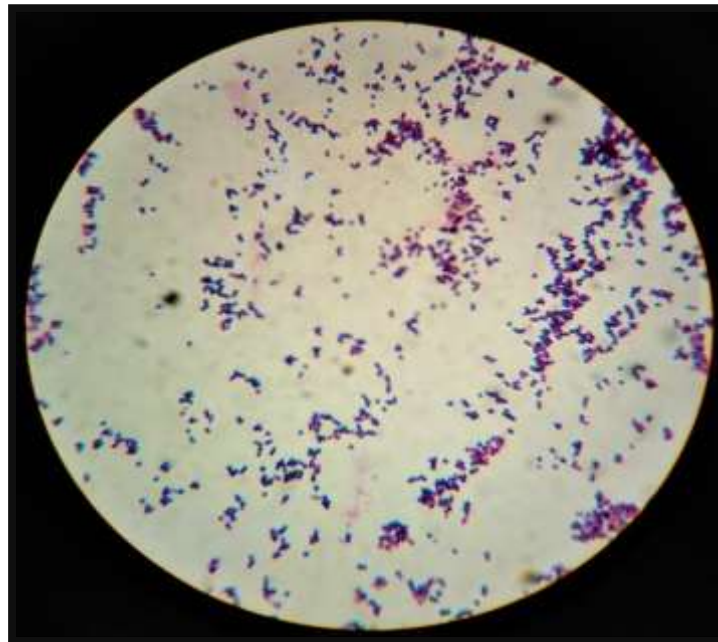
This study was approved by the Iraqi medical institutions and hospitals. Consent of all patients and healthy controls was approved to conduct this study and collection of sera samples. The identity of all patients was kept confidential as requested.

Results:

The results showed that among 90 samples collected, 83 isolates had basic characteristics of *Streptococcus* spp. Table 1 shows the biochemical test and morphological characteristics carried out on isolates that revealed typical characteristics of Streptococcal species in terms of types of hemolysis and Gram stain. 83 isolates tested positive to Gram stain (Figure 1) and blood hemolysis on blood agar plates. Among 83 isolates, 3 isolates showed α -hemolysis and transparent white colonies indicating that the isolates were *S. pluranimalium* (Figure 2). The biochemical tests of *S. pluranimalium* revealed negative to catalase and oxidase, small white colonies with greenish pigment indicating α -hemolysis of colonies and negative to Voges-Proskauer test. Arginine fermentation tests were found to be positive. Other sugars, such as sucrose, glucose and galactose, were also found to be positive, except in isolate 2 which showed negative to sucrose. Whereas mannitol was found to be negative. Furthermore, it has been found that *S. pluranimalium* was positive to esculin hydrolyzing test except in isolate 3 which was found to be negative to esculin hydrolyzing test (Table 1).

Table 1- Biochemical tests and morphology of *Streptococcus pluranimalium* isolates

Biochemical Tests and Morphology	Isolate 1	Isolate 2	Isolate 3
Oxidase	Negative	Negative	Negative
Catalase	Negative	Negative	Negative
Voger-Proskauer	Negative	Negative	Negative
Hemolysis	α -hemolysis	α -hemolysis	α -hemolysis
Arginine fermentation	Positive	Positive	Positive
Sucrose fermentation	Positive	Negative	Positive
Glucose fermentation	Positive	Positive	Positive
Mannitol fermentation	Negative	Negative	Negative
Galactose fermentation	Positive	Positive	Positive
Esculin hydrolyzing	Positive	Positive	Negative

**Figure 1-** *Streptococcus pluranimalium* under light microscope showing purple color cells in cocci shapes indicating a Gram positive.**Figure 2-** Growth of *Streptococcus pluranimalium* showing α -hemolysis and needlelike white colonies.

Molecular identification analysis showed that 3 isolates of *S. pluranimalium* tested positive to 16S rRNA sequencing. Figure 3 shows gel electrophoresis of PCR amplicons produced by 16S rRNA specific primers. The amplicons were then purified from agarose gel and sent for gene sequencing. The sequencing results showed high similarity to *S. pluranimalium* when blasted with gene bank database with close match of 99% sequence identity (Figure 4).

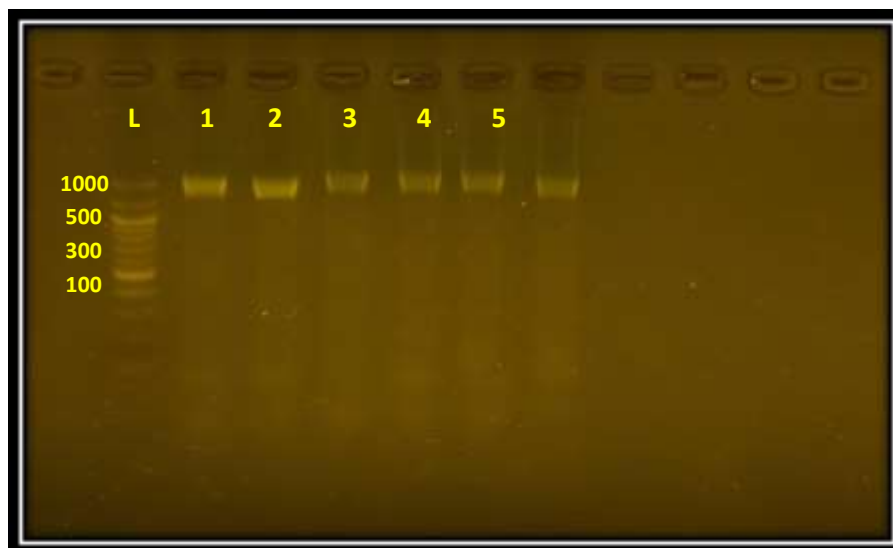


Figure 3-Agarose gel electrophoresis of 16S rRNA amplicons amplified from *S. pluranimalium* genomic DNA. L: 1kb DNA Ladder; 1 and 2: 1480 bp of 16S rRNA amplicon from isolate 1; 3 and 4: 1480 bp of 16S rRNA amplicon from isolate 2; 5 and 6: 1480 bp of 16S rRNA amplicon from isolate 3.

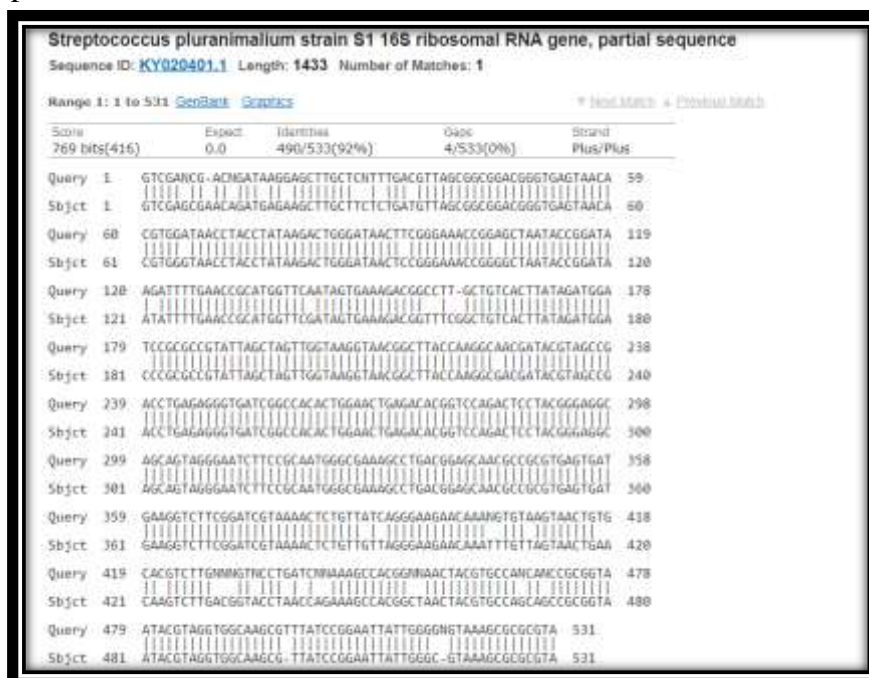


Figure 4: Alignment of 16S rRNA gene of *Streptococcus pluranimalium* with NCBI gene bank database showing sequence identity match of 92%.

Antibiotic susceptibility test revealed that *S. pluranimalium* had shown diverse response against the antibiotics used in this study. Table 2 shows antibiotic susceptibility test of *S.*

pluranimalium. As can be seen from Table 2, *S. pluranimalium* showed 100% resistance against azythromycin and erythromycin. However, the resistance percentage of *S. pluranimalium* was shown to be 67% against chloramphenicol and vancomycin. On the other hand, it was shown that cefotaxime and ceftriaxone were relatively effective against *S. pluranimalium* with 33% resistance percentage. Furthermore, *S. pluranimalium* was shown to be 100% susceptible against penicillin, levofloxacin and ofloxacin.

Table 2- Antibiotics susceptibility test of *Streptococcus pluranimalium*

Number of isolate	OFX	LEV	P	CRO	CTX	DA	VA	C	E	AZM
1	S	S	S	R	R	I	R	R	R	R
2	S	S	S	S	S	S	R	R	R	R
3	S	S	S	S	S	S	S	I	R	R

AZM: Azithromycin, E: Erythromycin, C: Chloramphenicol, VA: Vancomycin, DA: Clindamycin, CTX: Cefotaxime, CRO: Ceftriaxone, P: Penicillin, LEV: Levofloxacin, OFX: Ofloxacin, R: Resistant, I: Intermediate, S: Sensitive (CLSI, 2017).

Discussion:

S. pluranimalium is comparatively new and an uncommon pathogen that causes infections in animals and humans. It was first isolated in 1999 and was reported as new member of *Streptococcus* spp. [1]. Many study cases reported that this pathogen can cause number of infections in humans such as brain abscesses [17], endocarditis [18], cerebral abscess [7] and septicemia [1]. Although *S. pluranimalium* is reported as new pathogen, it is vital that more extensive studies are needed to characterize and identify *S. pluranimalium* virulence factors [12]. As the pathogen can be isolated from both animals and humans, it can be concluded here that the most prone people to *S. pluranimalium* infections are in contact with agricultural and domesticated animals. However, most diagnostic processes might misidentify this pathogen as other Streptococcal species. As a result, this might be challenging for healthcare sectors to treat this pathogen and thus understand its epidemiology and pathogenicity. Therefore, it is vital to identify this pathogen using more advanced identification methods such as 16S rRNA sequencing, real time PCR and Vitek identification process [19, 20].

In this study *S. pluranimalium* was isolated for the first time from infants and children who suffered from upper respiratory infections. Complete genome sequencing revealed that this pathogen possesses a variety of virulence factors such as hemolysin, sortase, IgA1 protease and some antibiotic resistance genes [12]. *S. pluranimalium* has β -galactosidase, arginine hydrolyzing enzymes and alkaline phosphatase [19-21]. The results of this study revealed that *S. pluranimalium* is positive to galactose and arginine in line with other studies [4, 8, 12].

16S rRNA sequencing based identification showed a very close match to the subject sequence when aligned with gene bank performed on NCBI database. This identity match provided a solid proof that these isolates belong to *S. pluranimalium* as previous studies had proved that 16S rRNA sequencing is a reliable test to identify *S. pluranimalium* [4, 7, 12, 18].

Antibiotic susceptibility test revealed a diverse response of *S. pluranimalium* against antibiotics used in this study. It was found that this pathogen is sensitive to penicillin, levofloxacin and ofloxacin. This susceptibility reason might refer to *S. pluranimalium* as being a natural host of animals. This means that *S. pluranimalium* might be sensitive to many antibiotics that were first used to treat the infection as the pathogen naturally causes infections in animals. One of studies showed that *S. pluranimalium* has some antibiotic resistance genes such as *mefA*, *msrD* and *lnuC* [12]. The *mefA* gene encodes for antibiotics efflux pumps that are responsible for inhibiting macrolides and lincosamides antibiotics. The *msrD* gene was found to be a member of ATP binding cassette proteins that mediates macrolides resistance. The *lnuC* gene was reported as being a lincomycin resistance gene. Study cases of *S.*

pluranimalium revealed that this pathogen is sensitive to vancomycin, carbapenem, aminoglycosides, clindamycin, ceftriaxone and 3rd generation of cephalosporins [7, 8, 17]. Another study showed that all bacterial isolates are resistant to erythromycin which is in line with this study findings [22].

It can be concluded that *S. pluranimalium* is a new pathogen that can cause very dangerous infections. Misidentification of this pathogen might be life threatening as *S. pluranimalium* not been properly studied. It can also be concluded that people in contact with domesticated and agricultural animals must be careful when dealing with animals as all studies reveal that *S. pluranimalium* is an animal pathogen that can be easily transmitted to humans causing infections.

Acknowledgements:

This study is supported by the Department of Biology, College of Education for Pure Sciences, University of Mosul. Many thanks to all participants, patients. A great thankful Central Laboratories/ Nenawa Health Office including doctors and nurses who were supportive to accomplish this study.

Conflict of interest: The author declares no conflicts of interest.

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