Isolation and identification of Streptococcus pneumoniae in children in the city of Mosul, and use of an innovative medium for preserving and transporting it

Saud Ameer Saadullah
Department of Biology, College of Science, Tikrit University, saud.a.saad4416@st.tu.edu.iq

Sundus Jassim Mohamad
Department of Biology, College of Science, Tikrit University, dr.sundus2017@tu.edu.iq

Dr. Firas Shawqi Algburi
College of Medicine, Tikrit University, dr.firas.shawki@tu.edu.iq

Abstract

The study included investigating Streptococcus pneumoniae infection among children in the city of Mosul, investigating the ability of pneumococci to secrete the Hyaluronate lyase, study the kinetics of this enzyme and the investigating of the inhibitory activity of some chemicals towards the activity of the enzyme. The study included (200) cases of children suspected of having Pneumococci distributed between (145) males and (55) females from different hospitals in the city of Mosul for the duration of (November 2021 until March 2022). S.pneumoniae was isolated and diagnosed by culture on brain heart infusion broth, Mueller-hinton agar and blood based agar medium. Then the isolates were purified and diagnosed according to phenotypic characteristics on the culture media, hemolytic phenotype, sensitivity to the antibiotic optochin, solubility in bile salts, agglutination test for pneumococcal antibodies and biochemical tests confirmed diagnosis by using VITEK 2 system. The study showed that out of the total of the suspects (47) cases were confirmed to be infected with pneumococci as resemble (23.5%). The samples taken from them varied and included (61.71% nasopharyngeal swabs, 25.53% acute otitis media, 2.12% cerebrospinal fluid, 0% blood and 10.64% sputum). The results of the antibiotic sensitivity examination of the isolates showed that they were (100% are sensitive to the levofloxacin, meropenem and vancomycin), (100% are resistant to ampicillin, cephalexin and azithromycin). (87.23%) are sensitive to ceftriaxone, (82.98%) are sensitive to chloramphenicol, As for the trimethoprim-sulphamethoxazole, the results showed that (19.14%) were sensitive, tetracycline (19.14%), and (19.51%) are sensitive to clindamycin, penicillin sensitivity showed (65.95%), while augmentin was (93.61%) resistant.

Keywords: *Streptococcus pneumoniae, GGFB medium, Pneumonia, Pastorex® meningitis kit, Draughtsman phenomenon.*

INTRODUCTION

Streptococcus pneumoniae is the most common cause of severe respiratory infections in children and community-acquired pneumonia in adults in the world, especially in US (Weinberger et al., 2014). Pneumococcus is the most significant etiology of acute respiratory infections, which cause three to five million fatalities yearly in children under the age of five (Brueggemann, van Rensburg, et al., 2021). Levels of transport are strongly

connected with the prevalence of pneumococcal infection illness and rising antibiotic resistance. Age, immunological state, seasonal fluctuation, and socioeconomic factors can all have an impact on transmission (which is acute in babies and decreases with age), several other demographic characteristics (Bogaert et al., 2004). The preferred antibacterial agent is penicillin, while macrolides are the second most popular substitute. The rise of S.pneumoniae strains that are resistant to many antibiotics such as, penicillin, macrolides, and other antimicrobial drugs has grown to be a significant healthcare issue during the past 20 years (Schroeder & Stephens, 2016; von Specht et al., 2021). The goal of the current investigation was to use Pastorex meningitis kit and Vitek 2 system to isolate and identify S.pneumoniae patients (Feagins et al., 2020). Using disk diffusion techniques to examine S.pneumoniae antibiotic sensitivity to various antibiotics.

Materials and Methods

1- Samples collection

A total of (200) suspected patients collected samples included (nasopharyngeal swabs, acute otitis media swabs, sputum, blood and CSF) were collected from children suspected of having pneumococcal infection, and the ages of the infected were distributed between (a few days to 14 years) of both sexes included (145) samples taken from males and (55) samples from females. Samples were collected from children's hospitals located on both sides of the city of Mosul during the period from November 2021 to April 2022.

2- Isolation of S.pneumoniae

The specimens were inoculated on blood agar & Brain-heart infusion broth. BA plates were incubated in 37 ºC, presence CO2 (5-10%) with inverted place for 18-24 h. Then, colonies suspicious of alpha-hemolysis and typical morphology (color, edges, glistening and mucous) were treated with Gram's stain, catalase and sub-cultured on blood agar plates with optochin discs incubated again. The optochin sensitive isolates were sub-cultured to obtain pure growth.

3- Identification of S.pneumoniae

Samples examined by microscopic examination to examine the bacteria from the suspected colonies under microscope after dyeing it by Gram's stain to see the (pneumococcus cell shape and size), catalase test, morphological identification of colonies characteristics including (color, shape, size, edges, consistency), optochin sensitivity test, bile solubility test and more confirmatory test such as (agglutination latex test used Pastorex® meningitis kit and VITEK 2 system to identification of biochemical tests).

4- (GGFB)(Glucose, Glycerol. Fluconazole and Blood) medium

This medium can be used to transport or preserve bacteria. It composed of solutions that's mentioned in table (1-1) were prepared, sterilized and adjust the pH to 7.2±0.2. Plastic beads (2 mm) in diameter, (0.05 g) per bead with different colors, washed by detergent to remove the contaminates and soaked in (1) M HCl to neutralize the pH and dried it at 45 ºC. Then, autoclaved to sterilize and kept closed until it used (Feltham et al., 1978), added to the components and mixed well in volumetric flask 250 mL, then the medium degree at 45 ºC utilize to add (5%) of volume warm defibrinated whole human blood to the medium and mixed well. Next, dispense the broth medium GGFB into two types of vials (the transfer vial contains 5 mL of medium without beads, but the preservation vial including cryotube contains 1.5 mL of GGFB medium and the broth will cover the beads). Store the tubes at 4 °C & -190 °C until use (Tedeschi & Paoli, 2011), see (fig 1-1).

Table (1-1): The contents of (GGFB) medium per (100 mL), pH 7.2±0.2

No.	Material	Amount
1.	Blood	5 mL
2.	Fluconazole	2.5 mg
3.	Glucose	0.5 g
4.	Glycerol	30 mL
5.	Plastic beads	5 g

5- Antibiotic susceptibility test

Mueller-Hinton agar medium supplemented with 5% defibrinated human blood used to check the sensitivity of the pneumococci for selected antibiotics by (Kirby-Bauer) diffusion test. Take a swab of the previously prepared fresh bacterial suspension (after compared it with 0.5 McF) and inoculate the medium, Leave it for 5 minutes, and then place the selected antibiotic disks and incubate them at 37 °C, presence CO2 (in anaerobic jar 5-10% CO2) for 20-24 h with invert plates. After incubation is over, measure the diameter of the inhibition zones with a ruler and compare them with the CLSI, 2021 tables to determine the sensitivity or resistance to antibiotics (Isenberg, 2016).

Table(3-6): Differential and confirmatory antibiotics for Pneumococcus, indicating type, symbol, concentration and manufacturer

No.	Antibiotic	Symbol	Disk content (µg)	Inhibition zone diameter (mm)		Company/ Origin
				S*	R*	
1.	Optochin	OP	5	≥14	<14	Bioanalyse/Turkey
2.	Oxacillin	OX	1	Depending on oxacillin sensitivity result, if (≥20 mm) it may consider sensitive to β-lactam group, if (<20 mm) MIC should work.		Himedia/India

Table (3-7): The antibiotics selected, indicating the type, symbol, concentration, and manufacturer

No.	Antibiotic	Symbol	Disk content (µg)	Inhibition zone diameter (mm)			Company/ Origin
				S	I*	R	
1.	Azithromycin	AZM	15	≥18	17-14	≤13	Bioanalyse/Turkey
2.	Ampicillin	AM	25	Depending on oxacillin sensitivity result, if (≥20 mm) may consider sensitive to β-lactam group			
3.	Ceftriaxone	CRO	10				
4.	Cephalexin	CL	30				
5.	Chloramphenicol	C	30	≥21	---	≤20	Rpicorp/USA
6.	Clindamycin	DA	10	≥19	18-16	≤15	Bioanalyse/Turkey
7.	Levofloxacin	LEV	5	≥17	16-14	≤13	
8.	Meropenem	MEM	10	If oxacillin sensitivity result (≥20 mm) may consider sensitive to β-lactam group			
9.	Penicillin G	P	10 U				
10.	Tetracycline	TE	30	≥28	27-25	≤24	Rpicorp/USA
11.	Trimethoprim-Sulphamethoxazole	SXT	1.25/23.75	≥19	18-16	≤15	Bioanalyse/Turkey
12.	Vancomycin	VA	30	≥17	≤16		

Results and Discussion

1- Isolation of S.pneumoniae

Two-hundred clinical samples were collected from children suspected of having pneumococcal infection based on the initial clinical diagnosis by the physician. The total number of positive infections with pneumococcal infection was (47) cases (23.5%) out of the total 200 cases, distributed among (33) males (16.5%), and (14) females (7%), see (fig 1-1). The results of body sites samples distributed between (nasal swabs 46.81%, AOM swabs 25.53%, throat swabs 14.9%, sputum 10.64%, blood 0 and CSF 2.21%) samples, see (fig 1-2). These results came close to results of Saleh., (2018) mentioned that the ratio for isolating pneumococcus was 27% from patients, for this percentage is considered close as it included all ages. The total percentage of confirmed pneumonia among children up to 14 years old in Nineveh governorate for the year 2021 was (2.56%), out of the total number of chest infections (52304), according to No. ICD-10 (J12-J18) of the statistics unit of the Nineveh health directorate, these results are reasonable and compatible with what we have reached.

Figure (1-1): Shows the total number of patients, the number of infections, and the percentages of pneumococcal infection for

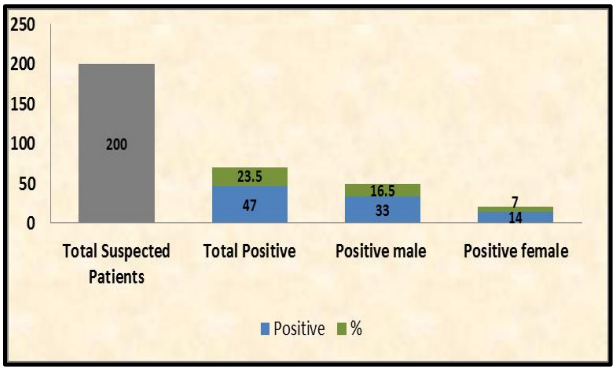
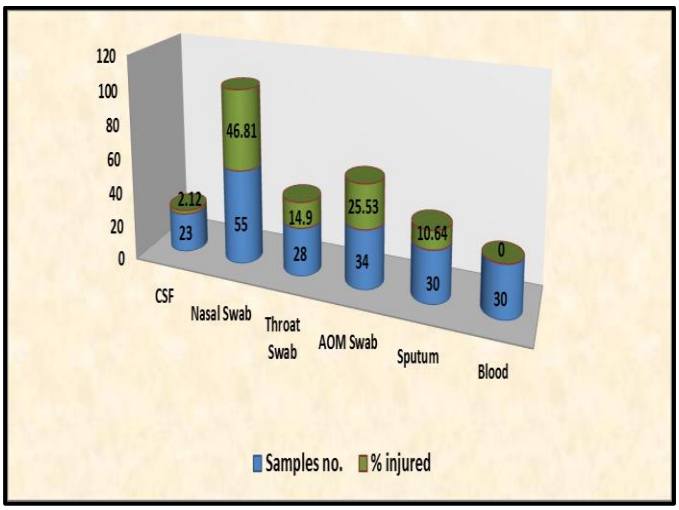
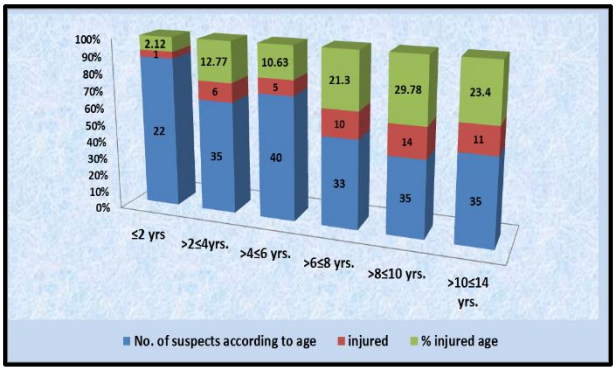


Figure (1-2): Shows the type of samples, their distribution, and the percentage of pneumococci isolated from the body sites.



The percentage of injuries varies among children with different ages, see (fig 1-3). The number of infections with pneumococci decreases for children under 4 years, and the reason for this is due to physiological, immunological and social reasons (Brealey et al., 2018). For children under 1 year of age, their immune system is in a state of slow development, and the IgA in milk colostrum gave protection especially for capsular bacteria (e.g., S.pneumoniae) and other fastidious, and IgG antibodies acquired will provide protection for them until the age of 6 months (Brooks & Mias, 2018).

Figure (1-3): Shows the age groups of pneumococcal injuries and the percentage of isolation from each category



These results are consistent with Wang & Dele Davoes, (2001). Also, children under two years of age are usually lived in a somewhat limited environment, unlike those older than 2 years who are in a wider environment that includes kindergarten and mixing with others which makes them acquire more aerosol droplets and another reason is related to not completing the pneumococcal vaccination (PPS 23) at the age of 8 years and over (Brueggemann et al., 2021). Most of the infections for 3 years are viral especially respiratory syncytial virus and rhinovirus or belong to other bacterial species such as E.coli, group B Streptococci and L.monocytogenes may be acquired since birth, and the extent of commitment to receiving complete vaccinations. While, increased in pneumococcal infections for groups aged 4 years and over. The results in the (fig 1-3) are somewhat consistent with the results of Abood et al., (2020) where there is an increase in pneumococcal infections at the age of 3-6 years at a rate of 23%. which are mostly due to S.pneumoniae, Staphylococcus aureus and other Streptococcus spp.

2- Identification of S.pneumoniae

Pneumococcal colonies on BA medium in presence of CO2 (5-10%) in mixed growth appear to be (0.5-1 mm) in diameter in size, dull gray, or watery like a tiny drop, produced narrow alpha-hemolysis around the colony (Jorgensen & Pfaller, 2015), see (fig 1-4). But, when isolated and re-cultivated in new BA plate appear to be (1.5-2 mm) in diameter, presence of heavy capsular around the colonies and more darkness of alpha-hemolysis appear underneath blood agar (Leber, 2020). The color produced by the hemolysis depends on the conditions of cultivation (Howden, 1976). In young colonies, it appears large in size, which soon becomes smaller with the age of the culture, shape are domed with a light white sticky apex and translucent margins but later it becomes flattened in center with raised edges looks like rings (draughtsman phenomenon) with the age of the colonies or in aerobic conditions, irregular and sometimes look circular when its separated (Willey et al., 2019), see (fig 1-5). The colony capsule appears dense in young colonies and tends to dry out with age, smooth-glial consistency, glistening or tear-like (it looks like light raindrops on the windshield of a car). This diagnosis was consistent with what both mentioned Rasheed et al., (2020).

Figure (1-4): Shows S.pneumoniae fresh colonies on BA medium with optochin disc sensitivity. Alpha hemolysis is light, opaque or translucent, irregular or circular colonies, some are flattened in the middle with raised edges small colonies appear (dome-shaped with a dense white head), glabrous or smooth in appearance. Noted draughtsman phenomenon for some colonies.

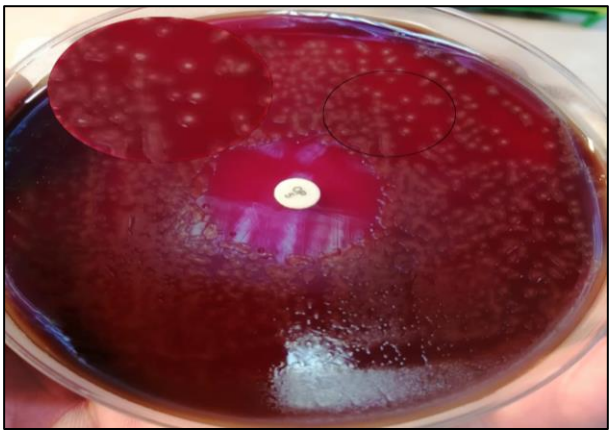
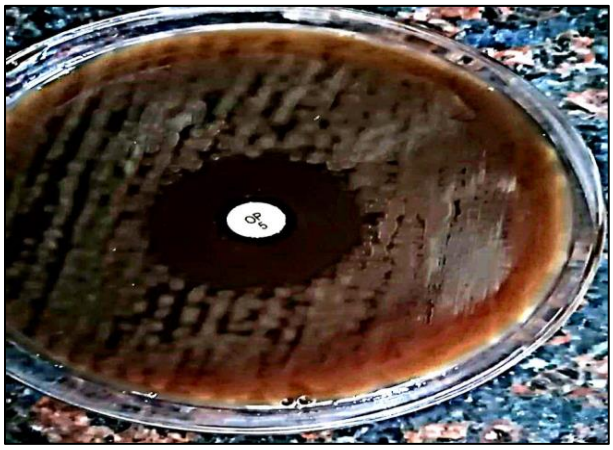


Figure (1-5): Shows S.pneumoniae aged colonies on BA medium with optochin disk sensitivity. Small, ring-shaped colonies with a concave center and raised edges by autolysis and dark alpha-hemolysis seems underneath the medium

On chocolate agar medium, pneumococcal colonies appear translucent on the surface with undulate edges, related to each other or undulate-circular when separated it, colonies on the medium look like inside the medium with chalky-slightly yellowish in precipitation like consistency, where the colonies appear as precipitated or suspended-within the agar (Tille, 2015), see fig (1-6).

Figure (1-6): Showing S.pneumoniae growth on chocolate agar medium with optochin disk sensitivity. Note the greyish-chalky-looking colonies that appear to have been deposited in the chocolate agar.



In BHI broth, pneumococcus grows in the form of fine threads after 18 hours of incubation, and these threads (microbial agglomerations) quickly disintegrate with the age of the culture due to autolysis, see (fig 1-7).

Figure (1-7): Shows colonies of S.pneumoniae cultured in BHI broth, looking like short threads. With the age of the culture, these threads quickly disappear due to the self-autolysis and become clear.

10 isolates were selected from the total number of S.pneumoniae isolates to confirm their diagnosis using a vitek 2 system GP card after they were diagnosed by optochin test. Tests were conducted in Al-Mansour laboratory in Mosul by using GP card, it gave a purity of (95% to 91%) for Pneumococcal isolates, and the evaluation of these results is very good, see (fig 1-8).

Figure (1-8): Biochemical tests of Vitek 2 GP card and results of S.pneumoniae isolate.

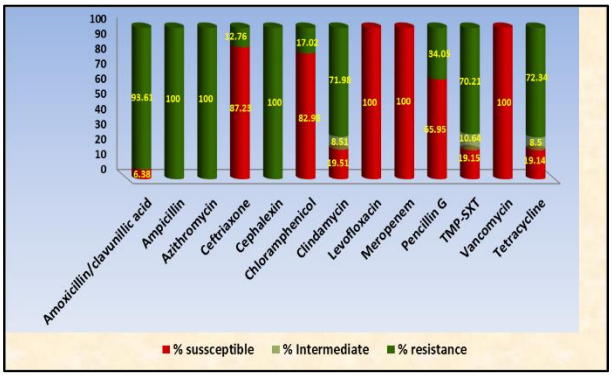
Well no.	Test	Result	Well no.	Test	Result	Well no.	Test	Result
2	AMY	-	4	PIPLC	-	5	dXYL	-
8	ADH1	-	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-
16	BGAR	+	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	-	24	BGURr	-
25	AGAL	+	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-
31	URE	-	32	POLYB	V	37	dGAL	-
38	dRIB	-	39	ILATk	-	42	LAC	V
44	NAG	v	45	dMAL	V	46	BACI	-
47	NOVO	-	50	NC6.5	V	52	dMAN	-
53	dMNE	-	54	MBdG	V	56	PUL	-
57	dRAF	+	58	O129R	-	59	SAL	-
60	SAC	v	62	dTRE	+	63	ADH2s	-
64	OPTO	-						

This system is fast, more frequent in tests, less expensive, and less material consuming than conventional systems such as API 20 Strep (Goessens et al., 2000).

Antimicrobial susceptibility test of S.pneumoniae

Antibiotics from different groups were used to check the sensitivity of pneumococcal isolates by using (Kirby-Bauer) disk diffusion method, by referring to the NCCLS recommendations and matching them with the CLSI guidelines (Humphries et al., 2021), and the possibility of some of them being given as treatment to children (Nelson, 2019), see (fig 1-9).

Figure (1-9): Diagram showing the results of the selected antibiotic susceptibility test for S.pneumoniae.



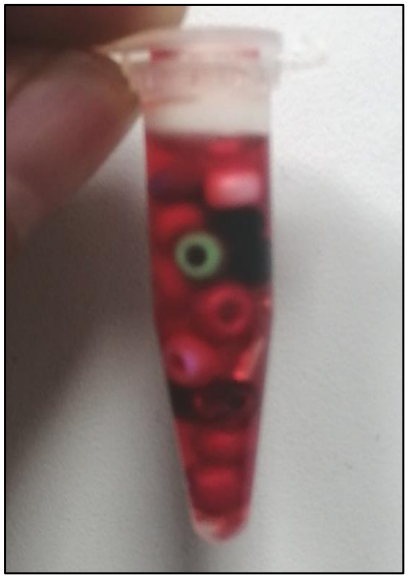
To investigate the sensitivity of pneumococci to penicillin, the antibiotic oxacillin was used as a preliminary test to investigate the sensitivity of the isolates to penicillin, and then to conduct sensitivity to penicillin after confirming the sensitivity of the isolate to oxacillin (Horna et al., 2016). The initial examination of the sensitivity test showed that (55.31%) of the isolates were sensitive to oxacillin, while (2.1%) gave a visual sensitivity of 12 mm to optochin. The results showed that all pneumococcal isolates (47) were sensitive (100%) to (levofloxacin, meropenem and vancomycin), while showed (100%) resistance for (ampicillin, azithromycin and cephalexin). It also showed a variation in the sensitivity of the isolates towards both ceftriaxone (87.23%), chloramphenicol (78.72%), penicillin (65.95%), trimethoprim-sulphamethoxazole (19.15%), tetracycline (17.02%) and clindamycin (19.5%), see (fig 1-9). These changes in susceptibility levels may be related to an increase MDR strains, acquisition of resistance genes especially in transformation because of misuse, lack of accuracy in diagnosing the condition, especially in the past two years with the outbreak of the corona pandemic. For penicillin's resistance, due to remodeling in PBPs related with mosaic structure in encoding genes (von Specht et al., 2021). In tetracycline resistance, these genes

play an important role in encoding efflux pumps the antibiotic out of the cell (Salsabila et al., 2022a). The results were close with the results of the Salsabila et al., (2022) in susceptibility of penicillin, chloramphenicol and vancomycin, also Motaweq & Naher, (2017) their results were sometimes close.

Evaluation of the (GGFB) medium in the transfer and preservation of S.pneumoniae

One of the most difficulties that faces while dealing with the fastidious bacteria (e.g., S.pneumoniae) is the possibility of isolating, transferring and preserving them, so it was necessary to think of a medium that encourages the isolation of S.pneumoniae and at the same time the possibility of using it to save samples. What distinguishes this medium is the presence of blood among its components, which provides a natural environment for the requirements of growth without there being a difference in the numbers of bacteria, as well as the high degree of preservation that does not allow the growth of bacteria and does not provide them with time due to the presence of other components within the medium. The contents for this medium exerts protection of specimens with the true number of bacteria in the samples with least growth factors. The purpose of using plastic beads to agglutinate bacterial cells during freezing and to provide a larger surface area for bacteria adhere to it, the presence of blood because of its components, which represent the ideal environment for bacteria and also use it for minimal source of nutrients, glycerol pass into the cell membranes and supply intra protection from freezing, glucose has a protective effect against extracellular freezing and fluconazole as antifungal (Tedeschi and Paoli, 2011; Nwosu, Abu and Agwa, 2019), see (fig 1-10).

Figure (1-10): GGFB medium in an Eppendorf tube.



Reference

Abood, S. F., Hussein, A. A., & Al-Salihy, S. A. R. (2020). Retrospective study of bacterial Infections among children under six years in Diyala governorate, Iraq. DYSONA-Life Science, 1(2), 76-82.

Bogaert, D., de Groot, R., & Hermans, P. W. M. (2004). Streptococcus pneumoniae colonisation: the key to pneumococcal disease. The Lancet infectious diseases, 4(3), 144-154.

Brealey, J. C., Chappell, K. J., Galbraith, S., Fantino, E., Gaydon, J., Tozer, S.,... & Sly, P. D. (2018). Streptococcus pneumoniae colonization of the nasopharynx is associated with increased severity during respiratory syncytial virus infection in young children. Respirology, 23(2), 220-227.

Brooks, L. R., & Mias, G. I. (2018). Streptococcus pneumoniae's virulence and host immunity: aging, diagnostics, and prevention. Frontiers in immunology, 9, 1366.

Brueggemann, A. B., van Rensburg, M. J. J., Shaw, D., McCarthy, N. D., Jolley, K. A., Maiden, M. C., ... & Zhou, F. (2021). Changes in the incidence of invasive disease due to Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis during the COVID-19 pandemic in 26 countries and territories in the Invasive Respiratory Infection Surveillance Initiative: a prospective analysis of surveillance data. The Lancet Digital Health, 3(6), e360-e370.

Clinical and Laboratory Standards Institute (CLSI). (2021). M100 Performance Standards for Antimicrobial Susceptibility Testing, Clinical and Laboratory Standards Institute (CLSI).

Feagins, A. R., Ronveaux, O., Taha, M. K., Caugant, D. A., Smith, V., Fernandez, K.,... & Wang, X. (2020). Next generation rapid diagnostic tests for meningitis diagnosis. Journal of Infection, 81(5), 712-718.

Feltham, R. K. A., POWER, A. K., PELL, P. A., & Sneath, P. H. A. (1978). A simple method for storage of bacteria at—76 C. Journal of Applied Bacteriology, 44(2), 313-316.

Goessens, W. H. F., Lemmens-den Toom, N., Hageman, J., Hermans, P. W. M., Sluijter, M., De Groot, R., & Verbrugh, H. A. (2000). Evaluation of the Vitek 2 system for susceptibility testing of Streptococcus pneumoniae isolates. European Journal of Clinical Microbiology and Infectious Diseases, 19(8), 618-622.

Horna, G., Molero, M. L., Benites, L., Roman, S., Carbajal, L., Mercado, E.,... & Ochoa, T. J. (2016). Oxacillin disk diffusion testing for the prediction of penicillin resistance in Streptococcus Pneumoniae. Revista Panamericana de Salud Publica, 40, 57-63.

Howden, R. (1976). Use of anaerobic culture for the improved isolation of Streptococcus pneumoniae. Journal of Clinical Pathology, 29(1), 50-53.

Humphries, R., Bobenchik, A. M., Hindler, J. A., & Schuetz, A. N. (2021). Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100. Journal of clinical microbiology, 59(12), e00213-21.

Isenberg, H. D. (2016). Clinical Microbiology Procedures Handbook. Clinical Microbiology Procedures Handbook.

Jorgensen, J. H., & Pfaller, M. A. (2015). Introduction to the 11th Edition of the Manual of Clinical Microbiology. Manual of Clinical Microbiology,14.

Leber, A. L. (2020). Clinical microbiology procedures handbook. John Wiley & Sons.

Motaweq, Z. Y., & Naher, H. S. (2017). Antimicrobial susceptibility of Streptococcus pneumoniae isolates causing LRTI in Najaf, Iraq. Environmental & Socio-economic Studies, 5(2), 10-18.

Nelson, J. D. (2019). Nelson's pediatric antimicrobial therapy 2020. American Academy of Pediatrics.

Nwosu, I. G., Abu, G. O., & Agwa, K. O. (2019). Isolation, screening and characterization of exopolysaccharide producing bacteria. Microbiology Research Journal International, 29(5), 1-9.

Rasheed, R., Hasni, K., Taj, M. K., Shafi, M., & Rasheed, S. (2020). Isolation and biochemical characterization of Streptococcus pneumoniae from throat swabs and sputum of children and adult in Quetta District. Pure and Applied Biology (PAB), 9(3), 1755-1762.

Saleh, M. B., & Jarullah, B. A. (2019). Isolation and Identification of Streptococcus pneumoniae isolated from Pneumonia patients in Thi-Qar province/Iraq. Journal of Education for Pure Science, 9(1).

Salsabila, K., Paramaiswari, W. T., Amalia, H., Ruyani, A., Tafroji, W., Winarti, Y.,... & Safari, D. (2022). Nasopharyngeal carriage rate, serotype distribution, and antimicrobial susceptibility profile of Streptococcus pneumoniae isolated from children under five years old in Kotabaru, South Kalimantan, Indonesia. Journal of Microbiology, Immunology and Infection, 55(3), 482-488.

Schroeder, M. R., & Stephens, D. S. (2016). Macrolide resistance in Streptococcus pneumoniae. Frontiers in cellular and infection microbiology, 6, 98.

Tedeschi, R., & Paoli, P. D. (2011). Collection and preservation of frozen microorganisms. In Methods in biobanking (pp. 313-326). Humana Press, Totowa, NJ.

Tille, P. (2015). Bailey & Scott's diagnostic microbiology-E-Book. Elsevier Health Sciences.

von Specht, M., Gabarrot, G. G., Mollerach, M., Bonofiglio, L., Gagetti, P., Kaufman, S.,... & Lopardo, H. A. (2021). Resistance to β-lactams in Streptococcus pneumoniae. Revista Argentina de Microbiología, 53(3), 266-271.

Wang, E. E., & Dele Davoes, H. (2001). Pneumonia in Children and Adolescents. Community-Acquired Pneumonia, 293-303.

Weinberger, D. M., Harboe, Z. B., Viboud, C., Krause, T. G., Miller, M., Mølbak, K., & Konradsen, H. B. (2014). Pneumococcal disease seasonality: incidence, severity and the role of influenza activity. European Respiratory Journal, 43(3), 833-841.

Willey, J., Sandman, K. M., & Wood, D. H. (2019). Encystment and Excystment. Prescott's Microbiolgy.