Assessment of Salicylic Acid Capability in Biofilm Disassembling of Local Penicillin Resistant -Streptococcus Agalactiae Isolated from Pregnant Women

Safaa Abed Latef Al Meani[1], MohammedMukhles Ahmed[2], Ali Hazim Abdulkareem[1]

[1]Assistant Professor, [2]Assistant Lect. Department of Biotechnology, College of Science, University of Anbar, Anbar, Iraq

Abstract

Streptococcus agalactiae, is the main aetiological agent of early neonatalsepsis in developed countries transmitted from pregnant women.*Twenty Streptococcus agalactiae* isolate collected from Vagina of pregnant women . All isolates were identified by routine methods , automated mehods and molecular method using 16S rRNA . All isolates gave a positive test result for CAMP tset, which is considered a differential test between *Streptococcus species.* β-hemolytic streptococci are

important human pathogens. GBS appeared decreasing in susceptibilityto penicillin with resistance ratio 15 % in Anbar city using disk diffusion agar method in the current study. ***Streptococcus agalactiae*** showed also resistant to Tetracycline (82%) , Clindamycin (60%) , Erythromycin (55%), chloramphenicol (40%) , Cipro (5%), Imipenem (0%) . Salicylic acid (SAL) reduced biofilm formation in ***Streptococcus agalactiae*** at statistical significance less than 0.001 compared with other materials .

Salicylic acid exhibited synergistic effect when combination by checkerboard technique with penicillin against ***Streptococcus agalactiae*** with activity fractional inhibitory concentration index0.41897.

Key words : *Penicillin- resistant , Streptococcus agalactiae , Salicylicacid , Synergistic effect.*

Introduction

Streptococcus agalactiae is the most common cause of human neonatalinfections. It is a significant cause of disease in pregnant women and seniors with underlying diseases such as diabetes or immunosuppression[1]. The organism colonizes 10–40% of pregnant women and is part of the gut and genital tract's natural flora. In adults and pregnant women, GBS can cause urosepsis, chorioamnionitis, endometries, pneumonia, skin and soft tissue infections. GBS causes newborn sepsis, diarrhea and meningitis[2][3]. However, human pathogenicity was not identified until 1938, when three reports of fatal post-partum infection were reported, Lancefield described GBS vaginal colonization for asymptomatic women [4][5]. *Streptococcus agalactiae* areconsidered to be susceptible in vitro to penicillin, although decreased susceptibility of penicillin was observed in Japanese isolates, which was considered to be secondary to reduced 2X expression of penicillin-binding protein (PBP). The first-line treatment for invasive GBS in adults is penicillin G[6]. In 2008, Kimura et al. published first GBSstrains with reduced susceptibility to penicillin. Further studies have confirmed that this decreased resistance is caused by mutations in the areas of penicillin-binding protein (PBP)[7] . Bacterial biofilms are complex organisms that adhere to biotic or abiotic surfaces, mono- or multi-microbial. The development of biofilms can be divided into several phases: The maturation and dispersion of the attachment as shown in Figure 1[8].

The largest metabolite of aspirin in vivo, salicylic acid, has been shown to interact with virulence factors in several viruses, including respiratory syncytial virus and HIV-1 transcriptional stage [9][10]. Aspirin, once ingested, becomes salicylic acid (SAL), the metabolite responsible for human anti-inflammatory, antipyretic, and antithrombotic properties[3]. We have recently shown that SAL strongly promotes S. aureus biofilm formation regardless of methicillin sensitivity or clonal genomic properties[11][12]. The current study aimed to isolate penicillin

-resistant *Streptococcus agalactiae* and know activity Salicylic acid against *Streptococcus agalactiae*.

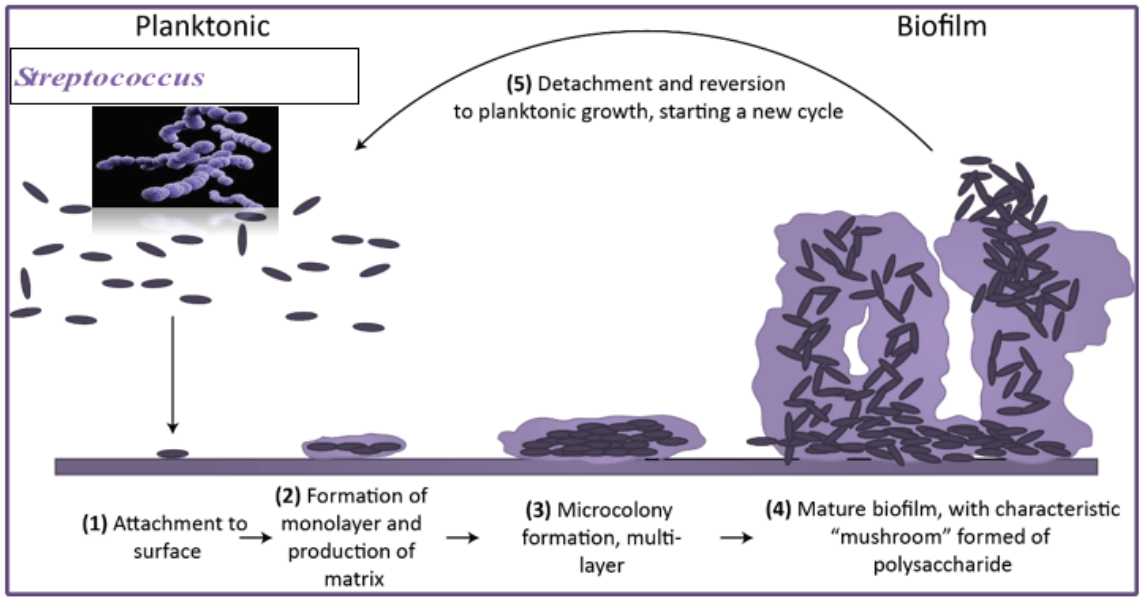

Figure 1 : Biofilm formation Steps in pathogenic bacteria cited by[35]

Materiales and Methods

Samples collection and bacterial diagnosis

The samples were taken from pregnant women vagina under aseptic condition . Twenty GBS clinical strains isolated from patients admitted to a Ramadi hospital in Anbar city of Iraq, during January–April 2020.

Identification of *Streptococcus agalactiae*:

Bacteria growing was diagnosed according to the protocol described in

[13]which includes morphological and microscopic examination and then confirmed with biochemical testes, automated test using Vitek-2 system , CAMP reaction and polymerase chain reaction based on 16S rRNA

CAMP reaction :

As previously described, isolates were tested for CAMP operation [14]. To summarize, the beta-toxin producing strain *S. aureus ATCC2522* was streaked on 5% sheep blood agar Tryptic soy plates.

Antibiotics susceptibility test of GBS

GBS antimicrobial resistance was measured using the disk diffusion method (Kirby-Bauer), as recommended by CLSI[9]. Clinical isolates were susceptible to seven different antibiotics, ndamycin, Chloramphenicol, Tetracycline, Erythromycin, Imipenem , Ciprofloxacillin, and Penicillin (Oxoid comp., UK).

Molecular Methods:

DNA was extracted by using Wizard^{R} Genomic DNA Purification Kit (Promega ; USA) according to manufacturer instructions. DNA concentration and purity were measured using a Nano-drop device . These primers were synthesized by Macrogen Co. ; Korea table 1. Full reaction of traitional PCR as shown table 2. The PCR was carried out in a25-µL volume (containing master mix [12.5 µL], yellow and blue loadingdye, and 2.5 µL of primer, 5 µL of the target DNA (10 ng), and nuclease-free water (2.5 µL).

Table1 : Sequence of 16 S rRNA.

Gene	Sequence (5' to 3')	Product size	Ref.	GenBank accession number
16S rRNA	F : CGCTGAGGTTTGGTGTTTACA	405	[15]	2353759
	R : CACTCCTACCAACGTTCTTC			

Preparation of Pharmaceutical Compounds :

One tablet of capecitabine was dissolved in 200 ml of warm water [16] ,200 mg Ibuprofen tablets were dissolved in the pH 7.2 100 ml phosphate buffer[17] . salicylic acid dissolved in water with sodium bicarbonate[18].

Determination of minimum inhibitors concentration :

Resazurin microtitre-plate assay (REMA) measured the minimum inhibitory concentration (MIC) antibiotic solutions with simple changes. Under aseptic conditions, 100 µl BHI broth which was contains 100 µl ofcapecitabine , Ibuprofen , and salicylic acid (separately) adding to the first row which containing 100 µl of BHI broth, and transfer 100 µl from the first row were transferred to the second row of the 96 well plates.

The material testing was conducted with a serial dilution of 100 µl, and then added 10 µl of bacterial suspension containing (10^{8} CFU / ml) to each well.

Biofilm formation :

Production of biofilm was measured using qualitative and quantitative assays, defined by Marques et al. ***Streptococcus agalactiae*** isolates were transferred to blood agar at 35°C for 24 hr [3] . The grown colonies were inoculated into tryptic soy broth (TSB) and studied chemicals (ibubrofen , salisylic acid, capecitabine) against biofilm development .

Synergism between penicillin and salisylic acid usingchechkerboard technique :

The possible presence of synergy interaction between the salisylic acid and penicillin was tested by the checkerboard method in 96 well microplates [19].

Statistical Analysis

Graph pad prism 8.0 was used in data analysis of this study . Paired T- test was used to analyze before and after treatment biofilm with ibubrofen , capecitabine , salicylic acid.

Results and Discussion

Isolation and Identification :

Streptococcus agalactiae appeared as glistening gray-white colonies with a narrow zone of beta hemolysis , When ***Streptococcus agalactiae*** isolates were cultured on Sheep Blood agar , while they appeared as orange coloured colony on grnada agar . Results of gram

Respe
stain, catalase, and oxidase were G+ dipococci , negative, and negative ctively. Figure (2).

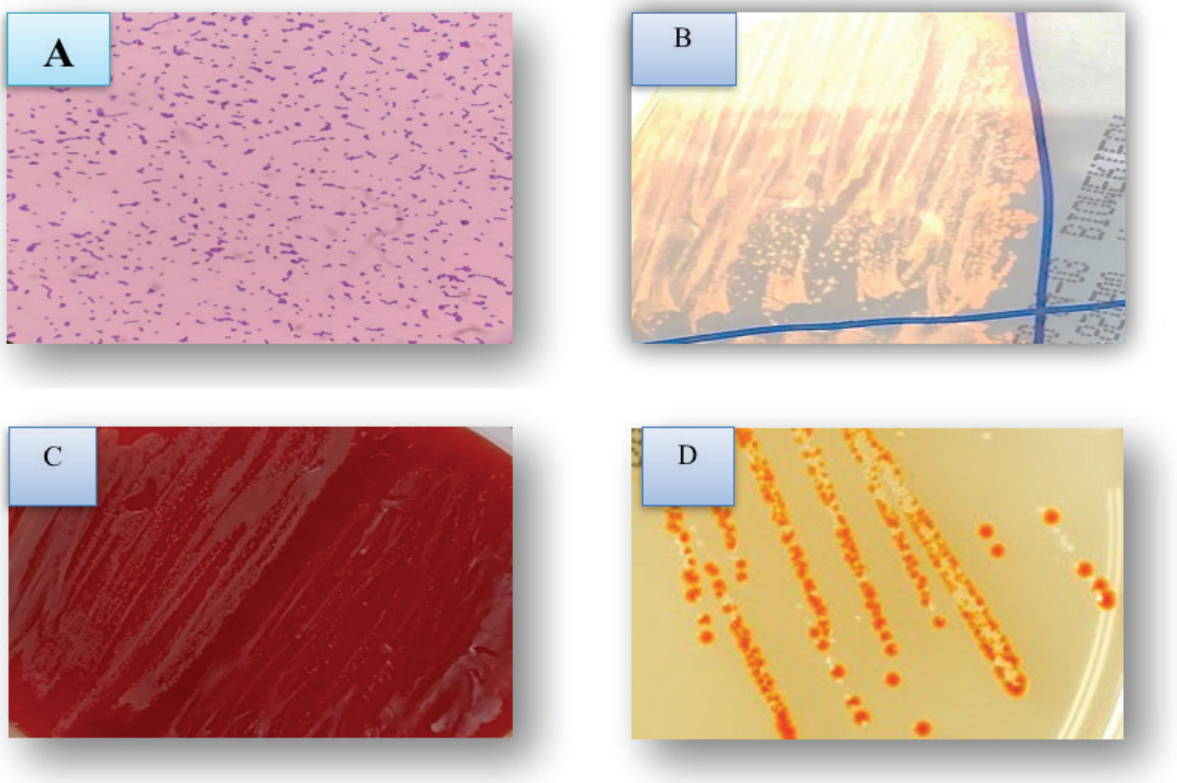

Figure 2 : Morphological and microscopic examination of Streptococcus agalactiae .
A: -Streptococcus agalactiae under microscope , B,D :
-Streptococcus agalactiae on grnada agar , C : -Streptococcus agalactiae on

CAMP confirmation Test :

CAMP test is used for the presumptive identification of group

B beta-hemolytic streptococci, *Streptococcus agalactiae*. All isolates gavepositive result with ratio 100%. As shown figure (3).

Although the CAMP factor lacks enzymatic activity, studies have shown that monomers can bind and oligomerize membrane components, most notably glycosylphosphotidylinositol (GPI)-anchored proteins, forming apore in the erythrocyte membrane[20]. According to the Koch postulates, Classification of the CAMP factor as a virulence factor remains controversial, as some authors have shown that injection of purified CAMP factor may increase rabbit and mouse mortality, whereas others have shown that deletion of the CAMP factor encoding gene (cfb) does not affect GBS pathogenicity [21]. Due to the widespread existence of the cfb gene in

GBS strains, the CAMP test or PCR check for the cfb gene wascommonly used to differentiate GBS from other Streptococcus species **[22]**.

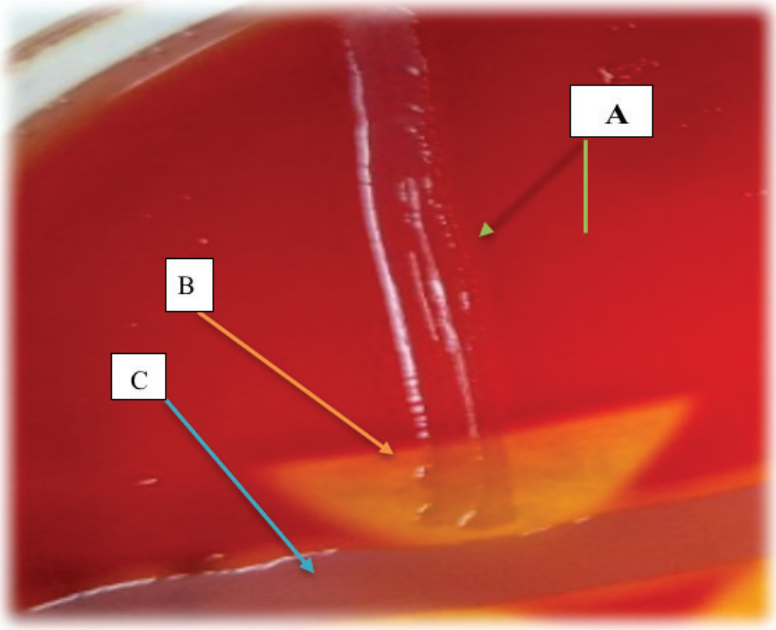

Figure 3 : CAMP test , A: Streptococcus agalactiae , B : positive result , C: S. aureus ATCC2522

Antimicrobial resistance testing of GBS :

According to figure 1 , results of disk diffusion for appeared that there are resistance to Tetracycline (82%) , Clindamycin (60%) , Erythromycin(55%) , chloramphenicol (40%) , penicillin (15%) , Cipro (5%) , Imipenem (0%) . figure 4, 5.

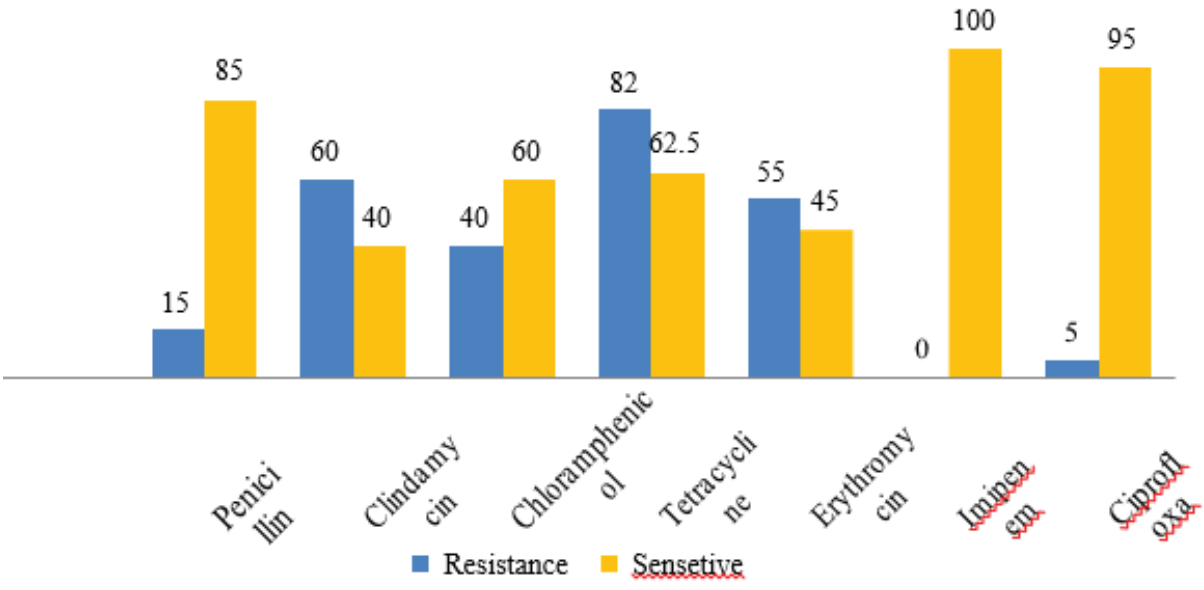

Figure 4 : Antibiotics disk ratio for Streptococcus agalactiae

However, since penicillin-resistant GBS strains have been discovered invarious parts of the world, including the United States of America [23], Africa [24], Colombia [24], Japan [25], Central Italy[26], Scotland[27], and Canada [27], Whether these resistance phenotypes are caused by spontaneous mutations acquired independently by some GBS strains, orby β-lactam-resistant GBS clones, or both, remains unresolved.

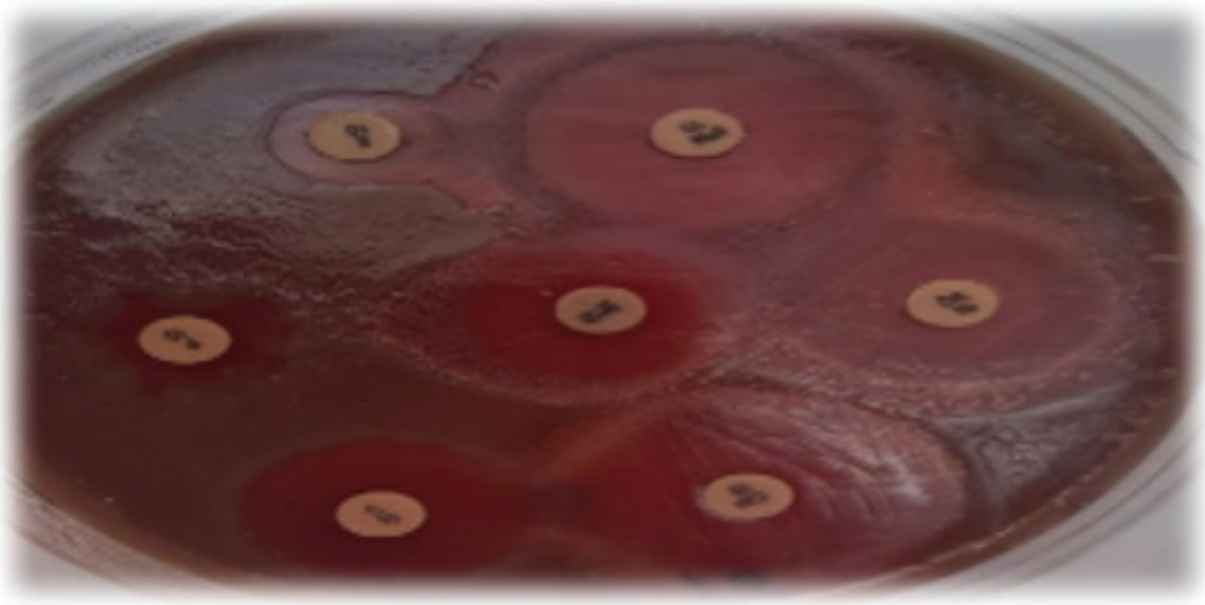

Figure 5: Disk diffusion of *Streptococcus agalactiae* on Blood agar

In Japan, the prevalence is high, at 2.3 percent in the period 2005–2006. Between 2012 and 2013, it increased to 14.7 percent. The above GBS with decreased penicillin susceptibility (PRGBS) were also multidrug resistant (MDR), with 71.1 and 95.6 percent resistance to erythromycin and levofloxacin, respectively, and 68.9 percent resistance to both antibiotics[28]. As a result, the spread of PRGBS with a proclivity for MDR has accelerated in Japan. The majority of PRGBS isolates were recovered from respiratory samples of elderly patients, while others were recovered from invasive samples of neonates and adults, according to Swedish and Japanese reports, but with only three PRGBS isolates in total [29].

In gram-positive bacteria, penicillin resistance is primarily caused by the development of altered, low-affinity target enzymes called penicillin-binding proteins (PBPs), which catalyze the terminal stage of bacterial cell wall peptidoglycan synthesis. The catalytic core of PBPs is formed by three conserved motifs, SXXK, SXN, and KT(S) G, which are frequently found in transpeptidase domains; and mutations inside or adjacent to these motifs are associated with their decreased affinity for β-lactams[30].

PBP2X and PBP2B both contain several amino acid substitutions. Thesemutations are associated with their penicillin minimum inhibitory concentrations (MICs). Recently, it was confirmed that GBS immune to ceftibuten but susceptible to penicillin was caused by amino acid substitutions in PBP2X.[31].

Molecular diagnosis of **All isolates gave apositive result for 16S rRNA as *Streptococcus***

Figure 6 : Molecular diagnosis of Streptococcus agalactiae uing 16S

Conclusion

In current work , salicylic acid(SAL) is suitable alternative agent against bacterial infection and anti-biofilm approach. The findings revealed that a significant percentage of GBS strains were immune to the most effective antibiotic. Given the increasing likelihood of finding penicillin-/ampicillin-resistant strains in the last period .In the fact that,Clinadamycin and erythromycin used in treatment streptococcus infection unstead of β-lactam antibiotics because of high resitance against this it.

Ethical Clearance: The Research Ethical Committee at scientific research by ethicalapproval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: Self-funding

Refernces

1. Savoia D, Gottimer C, Crocilla C, Zucca M. Streptococcus agalactiae in pregnant women: phenotypic and genotypic characters. Journal of Infection. 2008 Feb 1;56(2):120-5.
2. Manning SD, Lewis MA, Springman AC, Lehotzky E, Whittam TS, Davies D. Genotypic diversity and serotype distribution of group B streptococcus isolated from women before and after delivery. Clinical infectious diseases. 2008 Jun 15;46(12):1829-37.
3. Al Meani SA, Abdulkareem AH, Ibrahim MO, Ahmed MM, Mukhlif MY. Assessment the Correlation of D-dimer and Ferritin Level in Patients Infected with Covid-19 in Anbar Governorate of Iraq. Systematic Reviews in Pharmacy. 2020;11(9):1130-3.
4. Lancefield RC, Hare R. The serological differentiation of pathogenic and non-pathogenic strains of hemolytic streptococci from parturient women. The Journal of experimental medicine. 1935 Feb 28;61(3):335.
5. Al Maeni SA, Ahmed MM, Abed AD, Abbas RH, Jassim SA, Mohamad AM. Extraction and Purification of Staphylolysin Enzyme from Local Isolate of Pseudomonas Aeruginosa. Annals of the Romanian Society for Cell Biology. 2021 Apr 7:1201-8.
6. Ahmed MM, Al Meani SL. Occurrence of Klebsiella pneumoniae carbapenemase KPC gene in Klebsiella pneumoniaeisolated from patients in Anbar city of Iraq. Annals of Tropical Medicine and Health. 2019 Sep;22:108-16.
7. Kimura K, Suzuki S, Wachino JI, Kurokawa H, Yamane K, Shibata N, Nagano N, Kato H, Shibayama K, Arakawa Y. First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrobial agents and chemotherapy. 2008 Aug 1;52(8):2890-7.
8. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, Ali M, Rafiq M, Kamil MA. Bacterial biofilm and associated infections. Journal of the Chinese Medical Association.2018 Jan 1;81(1):7-11.

9. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science. 1994 Aug 12;265(5174):956-9.
10. Abdulkareem AH, Al Meani SA, Ahmed MM, Salih RM. Role of Potential Nanomateriales in Reducing Bacterial Resistance againstAntibiotics (A Review). Annals of the Romanian Society for Cell Biology. 2021 Apr 13:4023-38.
11. Dotto C, Lombarte Serrat A, Cattelan N, Barbagelata MS, Yantorno OM, Sordelli DO, Ehling-Schulz M, Grunert T, Buzzola FR. The active component of aspirin, salicylic acid, promotes Staphylococcus aureus biofilm formation in a PIA-dependent manner. Frontiers in microbiology. 2017 Jan 23;8:4.
12. Al Meani SA, Ahmed MM, Abdulkareem AH, Hamid NM, Ibrahim MO. Effect of COVID-19 Virus on Biomass Index of Infected Patients. Systematic Reviews in Pharmacy. 2020;11(9):1134-6.
13. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100. 2017.
14. Christie K, Atkins NE, Munch‐Petersen E. A note on a lytic phenomenon shown by group B streptococci. Australian Journal of Experimental Biology and Medical Science. 1944 Sep;22(3):197-200.
15. Dashtizade M, Zolfaghari MR, Yousefi M, Nazari-Alam A. Antibiotic susceptibility patterns and prevalence of streptococcus agalactiae rectovaginal colonization among pregnant women in Iran. Revista Brasileira de Ginecologia e Obstetrícia. 2020 Aug;42(8):454-9.
16. Meulenaar J, Beijnen JH, Schellens JH, Nuijen B. Slow dissolution behaviour of amorphous capecitabine. International journal of pharmaceutics. 2013 Jan 30;441(1-2):213-7.
17. Kumar RS, Yagnesh TN, Kumar VG. Optimisation of ibuprofen fast dissolving tablets employing starch xanthate using 23 factorial design. Int. J. Appl. Pharm. 2017;9:51-9.
18. Favorito JE, Eick MJ, Grossl PR. Adsorption of selenite and selenate on ferrihydrite in the presence and absence of dissolved organic carbon. Journal of environmental quality. 2018 Jan;47(1):147-55.
19. Langeveld WT, Veldhuizen EJ, Burt SA. Synergy between essentialoil components and antibiotics: a review. Critical reviews in microbiology. 2014 Feb 1;40(1):76-94.
20. Lang S, Xue J, Guo Z, Palmer M. Streptococcus agalactiae CAMP factor binds to GPI-anchored proteins. Medical microbiology and immunology. 2007 Mar;196(1):1-0.
21. Hensler ME, Quach D, Hsieh CJ, Doran KS, Nizet V. CAMP factor isnot essential for systemic virulence of Group B Streptococcus. Microbial pathogenesis. 2008 Jan 1;44(1):84-8.
22. Fuchs PC, Christy C, Jones RN. Multiple-inocula (replicator) CAMP test for presumptive identification of group B streptococci. Journal of clinical microbiology. 1978 Feb 1;7(2):232-3.
23. Dahesh S, Hensler ME, Van Sorge NM, Gertz RE, Schrag S, Nizet V, Beall BW. Point mutation in the group B streptococcal pbp2x geneconferring decreased susceptibility to β-lactam antibiotics. Antimicrobial agents and chemotherapy. 2008 Aug 1;52(8):2915-8.
24. Gizachew M, Tiruneh M, Moges F, Adefris M, Tigabu Z, Tessema B.Newborn colonization and antibiotic susceptibility patterns of Streptococcus agalactiae at the University of Gondar Referral Hospital, Northwest Ethiopia. BMC pediatrics. 2018 Dec;18(1):1-1.
25. Moroi H, Kimura K, Kotani T, Tsuda H, Banno H, Jin W, Wachino JI, Yamada K, Mitsui T, Yamashita M, Kikkawa F. Isolation of group B Streptococcus with reduced β-lactam susceptibility from pregnant women. Emerging microbes & infections. 2019 Jan 1;8(1):2-7.
26. Matani C, Trezzi M, Matteini A, Catalani C, Messeri D, Catalani C. Streptococcus agalactiae: prevalence of antimicrobial resistance in vaginal and rectal swabs in Italian pregnant women. Infez Med.2016 Sep 1;24(3):217-21.
27. Genovese C, D'Angeli F, Di Salvatore V, Tempera G, Nicolosi D. Streptococcus agalactiae in pregnant women: serotype and antimicrobial susceptibility patterns over five years in Eastern Sicily (Italy). European Journal of Clinical Microbiology & Infectious Diseases. 2020 Dec;39(12):2387-96.

28. Seki T, Kimura K, Reid ME, Miyazaki A, Banno H, Jin W, Wachino JI, Yamada K, Arakawa Y. High isolation rate of MDR group B streptococci with reduced penicillin susceptibility in Japan. Journal of Antimicrobial Chemotherapy. 2015 Oct 1;70(10):2725-8.
29. Persson E, Berg S, Bergseng H, Bergh K, Valsö-Lyng R, Trollfors B. Antimicrobial susceptibility of invasive group B streptococcal isolates from south-west Sweden 1988–2001. Scandinavian journalof infectious diseases. 2008 Jan 1;40(4):308-13.
30. Goffin C, Ghuysen JM. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiology and Molecular Biology Reviews. 1998 Dec 1;62(4):1079-93.
31. Pimentel BA, Martins CA, Mendonça JC, Miranda PS, Sanches GF, Mattos-Guaraldi AL, Nagao PE. Streptococcus agalactiae infection in cancer patients: a five-year study. European Journal of Clinical Microbiology & Infectious Diseases. 2016 Jun;35(6):927-33.