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Antimicrobial resistant among streptococcus agalactiae in AL-Hilla city

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(رَبِّ أوْزعني أَنْ أشْكُرَ نِعْمَتَكَ الّتي أَنْعَمْتَ عَلَيَّ وَعَلى والِدَيَّ وأنْ أعْمَلَ صالحِاً تَرْضهُ).

صدق الله العلي العظيم

الاحقاف من الاية ١٥

Dedication

To my father....

To my mother....

To my brothers....

To my sisters....

To my friends....

To my teachers....

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Abstract

In this study,120 vaginal and urine samples were collected from patients suffering from severe to moderate vaginitis and urinary tract

infection who attending to Babylon Hospital of Delivery and Maternal in hilla city through a period of three months. It was found that only seven isolates of *Streptococcus agalactiae* were identified. All isolates underwent culture and biochemical tests to confirm diagnosis, and it was reveled that the all isolates gave the same cultural and biochemical characters. However, other types of bacteria and yeasts were also isolated.

The effect of some antibiotics on *Streptococcus agalactiae* was investigated, and the results showed that all isolates were found that 42% of isolate are resistant to B-lactam antibiotic ,only 12.5 % are resistant to cephalosporine ,46 %are resistant to aminoglycoside , about 72% are resistant to macrolide and all isolate are resistant to cloramphenicol.

Some virulence factors of bacteria were also studied, and the results showed that all bacterial strains possessed capsules, which were regarded as the most virulent factor of the bacteria. The most characteristics of *Streptococcus agalactiae* is Gram-positive coccus which appears in chain or pair, catalase negative, facultivaly anaerobic bacteria, contain capsule and resistance to bacitracin. In respect of the ability of the isolates to produce Haemolysin, Siderophore, and Extracellular proteases, the results of the work showed that all the

isolates were able to produce bacterial haemolysin, but were not able to produce siderophore and extracellular proteases.

Chapter 1- Introduction & Literature review

1.1-Introduction:-

Streptococcus agalactiae or Lancefield group B Streptococci (GBS) is a species of genus *Streptococcus*. *Streptococcus* is part of the family of Streptococcaceae in the order of Lactobacillales. It is gram-positive cocci, catalase-negative and facultative anaerobic bacteria. It is divide in one plan ,therefore, its occur as pairs or chains. Their

metabolism is mainly fermentative and lactic acid is the predominant end product(Whiley et al., 2009). Streptococci with β-hemolysis on blood agar i.e. to lysate erythrocytes completely in blood agar are subdivided by their reaction to specific antisera against their group-specific cell wall anchored carbohydrate(Kilian, 2010) These tests were initially done with immunoprecipitation. This classification of these streptococci was described by Rebecca Lancefield. In this classification, *S. agalactiae* is the only species belonging to the serogroup B(Kilian , 2010)

Streptococcus agalactiae is part of the microbiota of the mucous membranes of humans and animals, mainly colonizing the intestinal, respiratory and genitourinary tracts (Whiley et al., 2009). First recognized as a pathogen causing bovine mastitis, Since the 1970s, the bacterium emerged as the leading infectious cause of early neonatal morbidity and mortality in the United States. The great medical importance of this microorganism is causing of severe septicemia, pneumonia and meningitis of neonates. It is also associated with urinary tract infections, postpartum endometritis, postpartum wound infection, septic pelvic thrombophlebitis and endocarditis. *S. agalactiae* is also an increasingly important cause of invasive infections in immunocompromised adults and the elderly.(Tsega et al., 2015) .

1.2- Virulence Factors of *Streptococcus agalactiae*

The factors that determine the initiation, development, and outcome of an infection involve a series of complex and shifting interaction between the host and the parasite, which can vary with different infecting microorganisms(Brogden et al., 2000). The microbial factors that contribute to the virulence of a microorganism can be divided into three major categories:-

1- Those that promote colonization of host surface.

2- Those that evade the host's immune system and promote tissue invasion.

3- Those that produce toxins that result in tissue damage in the human host.

Group B *Streptococcus* can produce many virulence factors associated with its pathogenicity. Some Group B *Streptococcus* virulence factors are secreted directly into the medium; others may be associated with the bacterial(Edwards,and Nizet,2011) *S.agalactiae* has the following virulence factor:-

1.2.1-Capsule:

GBS is usually encapsulated by a polysaccharide capsule. It is a major virulence determinant. There are nine serotype of polysaccharide capsule due to different polysaccharides that occur at different frequencies . Of the nine serotypes, the type Ia, Ib, II, III, and V are responsible for the

majority of neonatal human Group B *Streptococcus* disease. CPS types III and V as the most common followed by Ia, II and recently IV(Diedrick,et al., 2010) Serotype III which causes up to 50% of newborn infections, it causes a significant percentage of early-onset disease (infection occurring in the first week of life) and the majority of late-onset disease (infection occurring after the first week of life) in human neonates. It is also responsible for the majority (80%) of neonatal Group B *Streptococcus* meningitis cases . Type V is the most common capsular serotype associated with invasive infection in non-pregnant adults, and the emergence of type V strains over the past decade has been temporally linked to an increase in *Streptococcus agalactiae* disease in population .

1.2.2-Surface proteins

GBS express a variety of surface proteins, some of which are present in every strain such as the Sip or the FbsA protein. The FbsA protein is a surface exposed protein which binds to human fibrinogen and is therefore involved in the adhesion of GBS to human cells (Brochet,et al.,2008). The *sip* gene, encoding the surface immunogenic protein, is found in virtually all GBS strains. It was reported to promote colonization by enhancing adhesion, also an enhanced penetration of the blood-brain barrier by GBS in mice was reported.(Andreas 2012).

Other proteins are found in some but not all strains, and have been used for sero-subtyping purposes, most importantly the alpha-like protein (Alp) group(Lindahl et al., 2005). The Alps include six known protein designated Cα ,Alp1, Alp2, Alp3, Alp4 and R4. One of them is found in almost GBS strains but only very rarely more than one Alp is present. Alp4 originally detected in a bovine GBS strain, occurs infrequently and has to our knowledge never been encountered in human strains.

The C5a peptidase encoded by *scpB* cleaves the complement compon C5a ent . C5a which is a potent chemotaxin for polymorphonuclear leukocytes. Further the peptidase mediates binding of GBS to human immobilized fibronectin and has been shown to be involved in the invasion of epithelial cells by GBS. The lipoprotein encoded by *lmb* mediates binding of GBS to human laminin and thereby to epithelial cells . The *scpB* and *lmb* genes seem to be harboured by all human GBS, but is rarely found in bovine strains.

1.2.3-Pili

Recent studies demonstrate that GBS encodes small cell-surface appendages known as pili(Dramsi et al.,2006). Pili on the surface of bacteria are promoting adherence to epithelial cells. Other functions may

be to facilitate the formation of microcolonies and biofilms, mediate GBS resistance to AMPs and to promote transepithelial migration. GBS pili were found in 2005 by a reverse genetics approach (Dramsi et al.,2006)

1.2.4-Pore-forming toxins

Pore-forming toxins are a critical component of pathogenesis in many disease causing bacteria. These toxins promote entry of the pathogen into host cells and facilitate their intracellular survival and systemic dissemination. The virulence of pathogens defective in expression of pore-forming toxins is severely attenuated . GBS encodes at least two pore-forming toxins, known as β-hemolysin/cytolysin (β-H/C) and Christie Atkins Munch Peterson (CAMP) factor (Gonzalez et al.,2008).

1.2.4.1- Hemolysin:

Streptococcal hemolysins β-H/C, also known as CylE. It is surface associated protein can affect red blood cell and other kinds of cell and are better termed cytolysins. Its damage erythrocytes and hallmark phenotype of Group B *Streptococcus* in the clinical laboratory is the appearance of beta-hemolysis surrounding colonies growning on the surface blood agar plates.

β-H/C promotes GBS invasion of host cell barriers such as the epithelial and endothelial cells of the lung and the blood–brain barrier (BBB) and its regarded as an initial step in invasive disease. Hemolysin-

deficient GBS mutants are attenuated for virulence in various animal models of GBS infection, including sepsis, pneumonia, meningitis and arthritis (Hensler et al.,2008). Hemolytic activity of GBS is always associated with the synthesis of an orange pigment

In addition to direct cytotoxity, the Group B *Streptococcus* hemolysin exhibit proinflammatory, proapoptotic, and proinvasive properties that could contribute to disease pathogenesis. (Alkhalidy 2013).`

1.2.4.2-CAMP factor (Christie Atkins Munch Peterson)

CAMP factor is a secreted protein with pore-forming properties that has been important for GBS pathogenesis . Evidence that CAMP factor is important for virulence of GBS was provided by *in vivo* studies, which indicated that partially purified CAMP factor was lethal to rabbits, and co-administration of CAMP factor along with a sublethal dose of GBS can induce septicemia and death in mice. In addition, CAMP factor was observed to oligomerize and form discrete pores on susceptible target membranes . CAMP factor acts synergistically with the beta-lysin of *Staphylococcus aureus* to cause enhanced lysis of red blood cells, referred to as the CAMP reaction. The CAMP test was found to be a simple, rapid and reliable means of presumptive identification of GBS(Hensler et al., 2008).

1.2.5-Extracellular Protease Production:

Several lines of evidence indicate that the protease is important in the pathogenesis of Group B *Streptococci*. A cell-surface-associated protein (CspA) has been identified as an extracellular surface associated protease. The protein can cleave human fibrinogen and selected chemotaxins. CspA has been shown to be important for GBS to be fully virulent. .(Andreas 2012) .

1.3 Clinical manifestation

Streptococcus agalactiae is part of the normal flora of the gut and genital tract and is found colonizing 10–40 % of pregnant women. It is a well known cause of invasive infections in neonates and pregnant women. It has been recognized as a significant pathogen in non-pregnant adults especially among patients with underlying conditions such as diabetes mellitus, malignancy,(Huang et al., 2006) cardiovascular and genitourinary abnormalities, , AIDS, renal dysfunction, and peripheral vascular disease. Relapse is not uncommon, with approximately 5% of nonpregnant adults eventually experiencing a second episode of group B streptococcal disease(Sendi et al.,2008). In newborns, GBS is the cause of neonatal sepsis, pneumonia, and meningitis. Group B streptococcal neonatal sepsis is more common in the setting of prematurity and prolonged rupture of the membranes. Neonates can acquire the organism vertically in utero or during delivery from the maternal genital tract. Urinary tract infections are a common manifestation of group B streptococcal disease and are observed in both

pregnant and nonpregnant adults. Other presentations of group B streptococcal infection include pneumonia, skin and soft-tissue infections, septic arthritis, osteomyelitis, meningitis, peritonitis, and endo-ophthalmitis. Group B streptococcus can cause acute destructive endocarditis which may require emergency valve replacemen(Nandyal ,2008)

1.4 Epidemiology

Group B streptococcal disease results in significant mortality in both neonates and adults. Group B streptococcal(Phares et al., 2008) neonatal sepsis occurs in 1.8-3.2 per 1000 live births. The mortality rate ranges from 9-47% in published reports, most studies find it to be approximately 20%. The mortality rate is highest in elderly patients with comorbid medical conditions. The incidence of group B streptococcal disease in neonates appears to be decreasing, while the rate in nonpregnant adults appears to be increasing. The incidence of early disease has decreased over the past decade, likely because of the CDC guidelines for the prevention of neonatal colonization with group B streptococci(Phares et al., 2008)

1.5 Immunity

Immunity is mediated by antibodies to the capsular polysaccharide and is serotype-specific. Several serotypes are known—Ia, Ib, Ic, II, III, IV, V, VI, VII, and VIII. The absence of antibody to group B

streptococci in infants is a risk factor for infection. Because antibodies to group B streptococci provide protection against disease in animal models, there is an ongoing interest in vaccination as an approach for reducing the incidence of group B streptococcal colonization in healthy women. The factors that have made this approach less attractive include problems related to access to vaccination by women of childbearing age and the emotion and possible litigation associated with vaccination during pregnancy (Nandyal 2008)..

1.6 Antimicrobial susceptibility

Group B streptococci (GBS) are the main cause of neonatal infections. Intrapartum antibiotic prophylaxis is recommended for colonized women to prevent neonatal GBS disease, and penicillin is recommended as the first-line antibiotic (Claire et al., 1999). *Beta lactam antibiotic such as* penicillin and ampicillin are the drugs of choice for prevention or treatment of *Streptococcus agalactiae* infections, macrolid antibiotic such as clindamycin and erythromycin are the recommended alternatives antibiotics for patients who are allergic to beta-lactam agents. In the majority of antibiotics susceptibility studies(Pettersson , 2008) . B-Lactam remains uniformly active against *Streptococcus agalactiae* although there are scattered reports of non susceptibility to penicillin or ampicillin. Beta-lactam antibiotics such as penicillin are bactericidal in which its inhibited bacterial cell wall synthesis by preferentially binding

to specific penicillin-binding proteins (PBPs) that are located inside the bacterial cell wall. Bacterial penicillin-binding proteins (PBPs) are typically involved in peptidogylcan biosynthesis and are the site of action for the β-lactam family of antibiotics such as penicillin (Dermer et al.,2004). Amoxicillin' is similar to the natural penicillins, though amoxicillin is slightly less active than penicillin G against *S. agalactiae*

Cefotaxime is a synthetic, broad-spectrum cephalosporin antibiotic for parenteral administration. Cefotaxime has in vitro activity against a wide range of gram positive such as *Streptococcus agalactiae* The bactericidal activity of cefotaxime results from inhibition of cell wall synthesis. Cefazolin is first-generation semisynthetic alternative therapy to penicillin for group B6tsq streptococcal infection(Lin et al.,2011). Ceftriazone and meropenem are as effective as penicillin G and more effective than cephalothin, refampicin, and vancomycin. The use of second and third generation cephalosporins and meropenem is recommended in case of penicillin G allergy (Dahesh et al., 2008).

Vancomycin is also used as potent antibiotic directed against gram-positive organisms. Its useful in the treatment of septicemia and skin structure infections. It is indicated for patients who cannot receive or who have failed to respond to penicillins and cephalosporins Vancomycin is the initial treatment of choice for Group B *Streptococcal* infection in the penicillin-allergic individual(Lin et al.,2011).

Macrolides have broad spectrum of activity against positive cocci and have a longer half-life which means that they can be taken only once or twice daily (Levinson , 2006). Lincomycin has bacteriostatic mode of action against mainly gram positive bacteria like *Streptococcus agalactiae, Staphylococcus aureus, Streptococcus pyogenes and Diplococcus pneumonia.* Its reported that *Streptococcus agalactiae* had resistance to lincomycin (Dahesh et al., 2008). Tetracycline inhibits bacterial protein synthesis by preventing the association of tRNA with the bacterial ribosome. In GBS, resistance to this antibiotic is nearly ubiquitous and is most frequently due to a protein encoded by *tet*(M) or *tet*(O), which protects the ribosome from the action the tetracycline. Less common *tet*(K) or *tet*(L) genes encode a tetracycline efflux pump. Tetracycline resistance remains a clinically significant determinant to the utility of tetracycline, doxycycline and minocycline. *Streptococcus agalactiae* causing human infections: genetic diversity and capsular switching. Tetracycline resistance genes are often found on the same mobile element as erythromycin resistance genes.

In addition to that, Gentamicin is also used for the treatment of Group B *Streptococcus* infection. However, some strains of Group B *Streptococcus* have shown resistant to such antibiotics. It is observed that gentamicin show synergy when used with penicillin for Group B *Streptococcus*. In neonates, the ill patient with sepsis and in certain situations, such as endocarditis, adding an aminoglycoside as a second

drug may be helpful. The possible benefit must be weighed against the toxicity of renal and eighth nerve dysfunction, particularly in elderly people . The benefit of 2-drug therapy for group B streptococci has not been proven in terms of a better clinical outcome compared to penicillin therapy alone. The aminoglycoside needs to be tested against the isolate because only sensitive isolates can provide synergy (Nagano et al.,2008).

Oritavancin ,telavancin and dalbavancin are lipoglycopeptide antibiotic, it is a synthetic derivative of vancomycin that inhibits bacterial cell wall synthesis by interfering with polymerization and cross-linking of peptidoglycan and disrupts bacterial membrane integrity that leads to cell death. It is indicated for acute, complicated skin and skin structure infections caused by susceptible gram-positive bacteria, including *Streptococcus agalactiae*(Schwope et al., 2010).

Chloramphenicol is a relatively simple molecule containing a nitrobenzene nucleus, and prevents peptide bond synthesis, with a bacteriostatic result. It acts by binding on the 50S ribosome subunit, where it blocks the action of peptidyl-transferase, thereby preventing peptide bond synthesis. It has been used in the treatment of bacterial meningitis since the drug achieves satisfactory concentration in the CSF. It is active against both Gram-positive bacteria and Gram-negative bacteria, but it is a potent and potentially toxic. Its toxicity renders it unsuitable for systemic use except in some circumstances (Yenga John Bolukaoto, 2014).

The quinolones target DNA gyrase and topoisomerase IV, which are involved in the supercoiling of bacterial DNA that is essential to cellular processes such as DNA replication and transcription. GBS were uniformly susceptible to quinolones until 2003, when three isolates highly resistant to multiple quinolones were described in Japan, due to double-point mutations in the QRDR of *gyr*A and *par*C (88). Also in a report from Japan, high resistant rates to quinolones were later observed, particularly among invasive disease in non-pregnant adults (31.4%) and significantly associated to serotype Ib. Another survey from the United States identified 5% of the isolates as being resistant to levofloxacin, and suggested that healthcare-associated spread had occurred, as an association with prior quinolone therapy was observed. (*Streptococcus agalactiae* causing human infections: genetic diversity and capsular switching)

1.7 Antibiotic Resistance in GBS

Antibiotic resistance is the ability of bacteria or other microbes to resist the effects of an antibiotic. It occurs when bacteria change in a way that reduces or eliminates the effectiveness of drugs designed to cure or prevent infections. The bacteria survive and continue to multiply causing more harm (Beith 2008) Antibiotic resistance has actually

become a serious public health problem worldwide. However, a large part of the problem is due to the massive use or misuse of antibiotics in the biosphere which has had serious consequences for the antibiotic guidelines for the treatment of infections (Beith 2008, Greenwood et al .,2007). Several studies, carried out at various centers around the world, have assessed the antimicrobial susceptibility profile and determined the mechanism of resistance in GBS. These studies, in accordance with guidelines, found that penicillin is the antibiotic of choice for the treatment of GBS infections, followed by ampicillin and the 1st generation cephalosporins.

The main resistance mechanism of penicillin and cephalosporins in *S. agalactiaen* are amino acidic modifications in *pbp* genes (D. Faccone, 2010). It have reported that there is an increase worldwide in the resistance to two most commonly used antibiotics for penicillin allergic patients with high risk of anaphylaxis namely macrolides (erythromycin) and linconsamide (clindamycin).

Resistance against Macrolide, antibiotics among *S. agalactiae* may be occurring through two mechanisms: through target site modification and through an active efflux pump(Dahesh et al., 2008). Target site modification is encoded by the erythromycin ribosome methylase (*erm*) genes [*ermB* and ermTR], conferring resistance to MLSB(macrolide-licosamide streptogramin B) antibiotics. Phenotypic expression of resistance can be inducible or constitutive. On the other hand, an active

drug efflux system that functions via a transmembrane pump encoded by the*mefA* or *mefE* gene is responsible for macrolides and group B streptogramins resistance only(Back et al 2012). Erythromycin-resistant isolates with the MLSB phenotype will usually display cross-resistance to clindamycin, whereas isolates with the M phenotype usually display only erythromycin resistance (Khan et al.,2011) .

Several erythromycin resistance determinants and genetic carrying elements in GBS strains have been reported such as *ermB, ermA/TR, mef(A), mef(E), orf, intTn* .

In GBS infections, clindamycin provide useful alternative therapy for penicillin-allergic patients, but an increase in the resistance to this alternative drug has also been observed. Clindamycin resistance in GBS is due to ribosomal translocation encoded by *linB* genes. In this case, the resistance mechanism is the methylation of the 23S binding site. If this occurs then the bacteria are resistant to both macrolides and lincosamides. As clindamycin is a less potent inducer of 23S rRNA methylase as mentioned in MLS, erythromycin-resistant strains may appear susceptible to clindamycin *in vitro*. However, resistance will be manifest *in vivo.* (YENGA JOHN BOLUKAOTO, 2014).

Production of aminoglycoside-modifying enzymes is the principal cause of resistance to aminoglycosides. The genes for these enzymes are often plasmid-mediated, located on transposons, and transferable from one bacterial species to another. Resistance to aminoglycoside

antibiotics may occur by alteration of the 30S ribosomal target protein, but also through alterations in cell wall permeability or in the energy dependent transport across the cytoplasmic membrane(Ghearrdi et al., 2007)

Tetracycline resistance occur via two major mechanisms of: efflux and ribosome protection. resistance to tetracycline is encoded by ribosome protection genes including *tetM* and *tetO* or by efflux pumps is encoded by the *tetK* or tetL genes. Tetracycline resistance genes are often found on the same mobile element as erythromycin resistance genes. Other mechanisms of bacterial resistance to tetracyclines include enzymatic inactivation of the drug and production of bacterial proteins that prevent tetracyclines from binding to the ribosome. Resistance to one tetracycline does not confer universal resistance to all tetracyclines.
Resistance primarily occurs via reduced binding at the target site.
Reduced susceptibility and resistance have been reported in *S.agalactiae.* Cross-resistance with other protein synthesis inhibitors does not occur.

The most common mechanism of chloramphenicol resistance involves the inactivation of the drug by a plasmid mediated enzymatic, mechanism which is easily transferable to bacterial population,(Mims *et al.*, 2004).

Fluoroquinolone resistance is a growing problem in human pathogens and *S. agalactiae* isolates with this phenotype have recently emerged in a few countries. The main resistance mechanisms known

today are mutations in the quinolone resistance- determining region (QRDR) of ParC protein in positions Ser79 and Asp83 (Beith . 2008 and Tazi et al.,2012). Additional mutationsin Ser81 and Glu85 of GyrA protein are also related to fluoroquinolone resistance. (D. Faccone, 2010) .

Resistance to the *trimethoprim–sulfamethoxazole* combination is less frequently encountered than resistance to either of the drugs alone, because it requires that the bacterium have simultaneous resistance to both drugs. Significant resistance has been documented in a number of clinically relevant organisms, including *S.agalactiae*.

Evolution of bacteria towards resistance has been considerably accelerated by selective pressure exerted by over prescription of drugs in clinical settings; self-medication; inappropriate antibiotic treatment; the failure of taking the entire prescribed course of antibiotics; and the use of antibiotics to treat viral infections. Those are among other factors that promote emergence of antibiotic resistance (YENGA JOHN BOLUKAOTO, 2014).

Chapter II:-Material and methods

2.1 Collection of samples

A total of 120 vaginal and urine samples were collected from patients suffering from severe to moderate vaginitis and urinary tract infection who attending to Babylon Hospital for Maternal and Delivery in hilla city through a period of three months. The samples were taken according to the methods suggested by (Collee *et. al.*,1996). In vaginitis: The swabs are inserted into the upper part of the vagina and rotated there before withdrawing it, so that exudate is collected from the

upper as well as the lower vaginal wall. An endocervical swab must be collected. A vaginal speculum must be used to provide a clear sight of the cervix and the swab is rubbed in and around the introitus of the cervix and withdrawn without contamination from the vaginal wall. Swab should be placed in tubes containing normal saline to maintain the swab moist until taken to laboratory. The swab has been inoculated on culture media and incubated aerobically for 24h. at 37°C. In urinary tract infections: specimens of urine was generally collected in plastic universal sterile containers

2.2 Bacterial identification

Streptococcus agalactiae was isolated and identified according to its characteristics and then compared with their characteristic being reported in referential references MacFaddin, (2000). The characteristics being investigated are:

A -Cultural characteristics:

A single colony was taken from each primary positive culture. Its identification depended on the morphology properties (colony size, shape, color, translucency, edge, and elevation of texture). A colony that is gray has been selected with beta hemolysis on blood agar.

B-Microscopic Examination: After staining the bacteria with gram stain, its specific shape, color, aggregation and specific interacellular

compound have examined. Under microscope, *Streptococcus agalactiae* ppears in pair or in chain and gram positive.

C-Biochemical test

Biochemical tests have been done to reach final identification according to Bergy's Manual For Determinative Bacteriology(Holt, *et.al.*, 1994). The following biochemical tests were performed for the identification of *Streptococcus agalactiae* isolates from other isolation:

1- Catalase Test:-

A small amount of bacterial growth which grow on medium in age 24hr. is transferred by sterile wooden stick onto the surface of a clean, dry glass slide, and one drop of (3%H2O2) is added to it. The formation of gas bubbles indicates the positive results. Some organisms (e.g. Staphylococcus aureus) produce a peroxidase that slowly catalyzes the breakdown of hydrogen peroxide with evolution of oxygen bubbles. GBS does not produce the peroxidase and so it is negative for catalase test.

2- Oxidase Test

A filter paper circle was placed into a sterile plastic disposable petridish and moistured with several drops of the freshly prepared oxidase reagent, then a small portion of the colony to be tested was removed and rubbed on the filter paper, changing the color to blue or

purple within10 seconds indicated for a positive result (Baron *et al.*,1994) .

3- Voges-Proskaure Test:-

The test is performed by using MR-VP broth. The inoculated media is incubated for 24hr. at 37°C. Afterwards, 15 drops of 5% alpha-nephthol are added and followed by 10 drops 40% KOH. The mixture is shaken well and allowed to stand up to 15 minutes before calling a reaction negative. If positive, the culture turns red at the surface of the liquid, and the color spreads gradually throughout the tube. The positive result indicates a partial analysis of glucose which produce(Acetyl-carbonyl) (Collee, *et.al.*, 1996).

4- Easculin test:

The organism is grown in esculin slant for 24hr. at 37°C. The dark brown color indicates a positive result. The unchanging

of the color is a negative result (Baron, *et.al.*, 1995).

5- CAMP Test:-

Inoculate a β-lysin producing of *Staphylococcus aureus* as a streak across a blood agar plat containing 5% sheep blood. Then inoculate a single streak of the *Streptococcus* perpendicular to that of *Staphylococcus*, leave 1 cm of space between the two streaks, and then incubated plate at 37°C for 24h. The positive result is appear an

arrowhead-shaped zone of enhanced hemolysis at the juncture between positive *Streptococcus* and the *Staphylococcus* (Collee, *et.al.*, 1996).

6- Coagulase Test:-

Several colonies of bacteria are transferred with a loop to a tube containing 0.5 ml of plasma. The tube is covered to prevent evaporation and incubated at 37°C overnight. The test is read by tilting the tube and observing for clot formation in the plasma. A negative test results in the plasma remaining free-flowing with no evidence of a clot (MacFaddin, 2000).

7- Haemolysin production

Hemolysin production was carried out by inoculating a blood agar medium with bacterial isolates at 37^{o}C for 24 hours. *Streptococcus agalactiae* form a clear zone around the colonies, that would refer to complete hemolysis (ß- hemolysis) (De Boy *et al*., 1980).

8 - Bacitracin test

Beta-hemolytic (Group B-streptococcus) are resistant to (not killed by) bacitracin, while Group A streptococci(beta-hemolytic) are sensitive to (killed by) the antibiotic bacitracin. A sterile disk impregnated with bacitracin is placed on the first sector of an isolation plate before incubation. A zone of inhibition (area with no growth) will be seen around the disk after incubation if the organism is a Group A beta-

hemolytic Streptococcus. Their colonies will thus grow right up to the disk of bacitracin.

2.3 Antibiotic sensitivity assay (Antibiotic diffusion tests)

This test was performed on Muller-Hinton agar the using the Kirby-Bauer methods (Bauer *et.al*.,1966). It was performed by using a pure culture of previously identified bacterial organism. The inoculums to be used in this test was prepared by adding growth from 5 isolated colonies grown on a blood agar plate to 5ml of nutrient broth. This culture was then incubated for 3-4hrs to produce a standard bacterial suspension of moderate turbidity equal to McFarland standard tube(0.5). A sterile swab was used to obtain an inoculums from the standardized culture. This inoculums was the streaked on a Muller-Hinton plate. The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps. Incubation was usually overnight with optimal time of 14 hours at 37ºC. Antibiotics inhibition zones were measured. Zone sizes was compared to standard to determine the susceptibility or resistance of organism to each antibiotic according to (CLSI, 2010) criteria.

Chapter III:-Results and Discussion

3-1. Diagnostic features of *Streptococcus agalactiae*

The most characteristics of *Streptococcus agalactiae* is Gram-positive coccus which appears in chain or pair, catalase negative, facultivaly anaerobic bacteria, contain capsule and resistance to bacitracin.

Streptococcus agalactiae is non-motile they are unable to synthesize heme compounds. *Streptococcus agalactiae* nutritionally fastidious with variable with nutritional requirements, and growth on complex media is enhanced by the addition of blood or serum. Glucose and other carbohydrates are metabolized fermentatively with production of lactic acid as a major metabolic end product. Gas is not produced as a result of glucose metabolism(Hardie, 1986).

On blood agar, GBS produce translucent to opaque, whitish gray, soft, smooth, small colonies that are moist. Most, but not all strains produce a relatively narrow zone of beta-hemolysis. The best medium for isolation and identification of *Streptococcus agalactiae* is Columbia agar. The orange colonies may be considered a primary diagnostic feature for the isolation of this bacteria (Mosabi, *et.al.*, 1997) and by this medium, it can differentiate this bacteria from other *Streptococci* which cannot give the same color on such medium.

Furthermore, Bacitracin (10 unit) is also used to differentiate this bacteria from group A *Streptococci* in that the latter is highly sensitive to this agent but the former GBS is resistant (Collee, *et.al.*, 1996).

Table(1) show the results of morphological and biochemical features being used for identification and isolation of *S.agalactiae* . The results were compared with referential reported by (Baron *et.al*.,1994),(Collee *et.al.*,1996) and MacFaddin,(2000).

(Table 1) Diagnostic features of *Streptococcus agalactiae*

TESTS	RESULS
Hemolysin	Beta (narrow zone)
Bacitracin	Resistance
Growth on blood agar	Gray color with narrow zone of hemolysis
Growth on Columbia	Orange color
Gram stain	Positive
Shape of cell	Coccus(chain or pair)
Catalase	Negative
Oxidase	Negative
Capsule	Positive
CAMP	Positive
Easculin	Negative
VP	Negative

3.2. Isolation and Identification of *Streptococcus agalactiae* .

A total of 120 samples including (urine and vaginal) was collected from patient attending to Babylon Hospital for Maternal and Delivery in

Hilla city during periods from september 2015 to December 2015 . All samples were subjected for culturing on available media and it was found out of the total of 120 samples, only 110 samples showed positive cultures. No growth was seen in the other samples (8 samples) which could indicate the presence of microorganisms Among 110 only 7 isolates of *St. agalactiae* were identified diagnosed with prevalence rate 5.8% (figure 1).

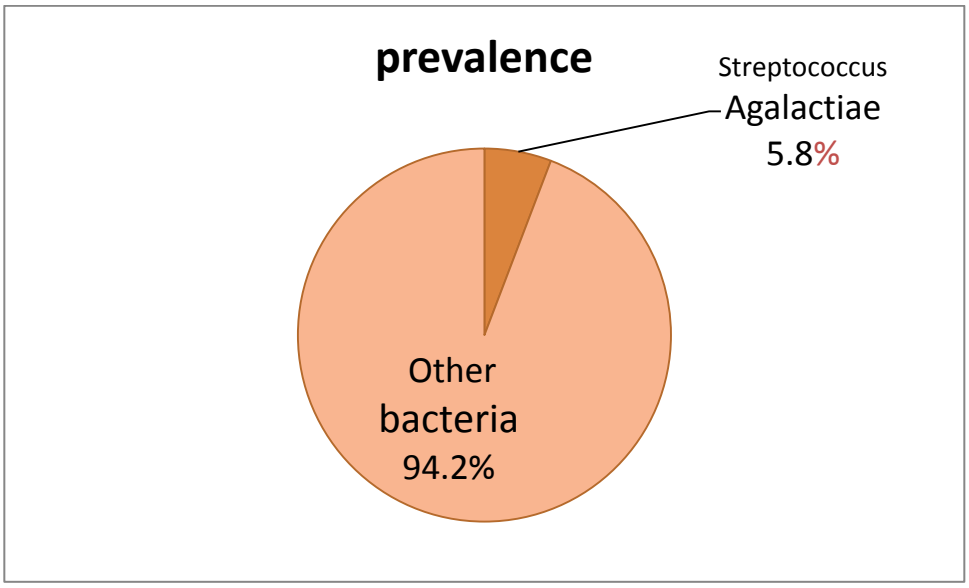

Figure (1) The prevalence of *St. agalactiae bacteria*

This result is correlated with the results of local study obtained by lamees (2005)where it was reported that prevalence of these bacteria was 6%. Alaq (2013) , indicated that among (55) vaginal samples only 5 isolates of *S. agalactiae* have been isolated from pregnant women, while 15 isolates *S. agalactiae* have been isolated from non pregnant women suffering from vaginitis .

Similar results obtained by other studies like (Suzanne *et al*.,2011) in whichs its found that the rate of prevalence of these bacteria among urine samples was (2.08%).

In study done by (Stephanie M Borchardt *et al*., 2006) its found that the prevalence of *S.agalactiae* among vaginal and urine samples was (17.4%) an (14.4%).(Masoumeh *et al*., 2015) found that among 2400 specimens collected, vagina specimens indicated a higher proportion of GBS infection (11.05%) among the rest of isolates.

(Tsega *et al*., 2015) stated that 11.3% of pregnant women had rectovaginal colonization with *S. agalactiae*. A comparable rate of colonization was also reported in the others study done in Gondar, Ethiopia (9%) and in Saudi Arabia (9.2%). This prevalence is within the often quoted range of between 5.2%-34.3% in different parts of the world .

However, (Edward and Baker,2000) have proved that the rate of isolation of *Streptococcus agalactiae* from vaginal swabs ranges from 5-40% due to difference in the sample sites and culture method employed. and generally to the geographic location or characteristics of the population investigated

The prevalence of GBS carriage in the vagina at the time of delivery varies from 5 to 30%, with peripartum transmission to the newborn

resulting incolonization in 50–70% of cases, if no action is taken to prevent transmission. (Suzanne *et al.*,2011).

GBS is identified as an infectious agent of invasive disease in non-pregnant adults especially those underlying conditions such as diabetes mellitus, malignancy, or liver disease. The incidence of invasive GBS infection in non-pregnant adults has increased four-fold recently up to 4.1-7.2 per 100.0003.

The presence of microorganism in the female lower genital tract (LGT) in the concentrations that modify the established normal equilibrium produces different symptoms that make necessary to consult the gynecologist. It is currently accepted that infections of the LGT are due to microorganisms that are normally integrating the internal flora, except for the erroneously called sexually transmitted disease that are caused by external microorganisms. This means that all those microorganisms usually present without causing any disease (Provenzano, 1999).

GBS also cause urinary tract infections (UTIs), which encompass asymptomatic bacteriuria, cystitis, pyelonephritis, urethritis, and urosepsis. GBS asymptomatic bacteriuria is particularly common among pregnant women; however, those most at risk for cystitis due to GBS are the elderly and immunocompromised individuals. Predisposing factors for GBS UTI may include diabetes mellitus and chronic renal failure. Between 5% and 23% of nonpregnant adults with invasive GBS disease

present with a urinary tract infection8. Clinical manifestations of GBS infection in adults are numerous and quite varied. Because group B streptococci may colonize skin and mucosal surfaces and may be isolated from infected sites along with other virulent organisms, their role in pathogenesis has often been questioned. (Homa *et al*., 2015)

3.3 Antibiotics sensitivity assay

The results of antibiogram profile showed that the most isolates of GBS were resistant to various degrees to one or more of antibiotics. It was found that 42% of isolate are resistant to B-lactam antibiotic ,only 12.5 % are resistant to cephalosporine ,46 %are resistant to aminoglycoside , about 72% are resistant to macrolide and all isolate are resistant to cloramphenicol (figure 2)

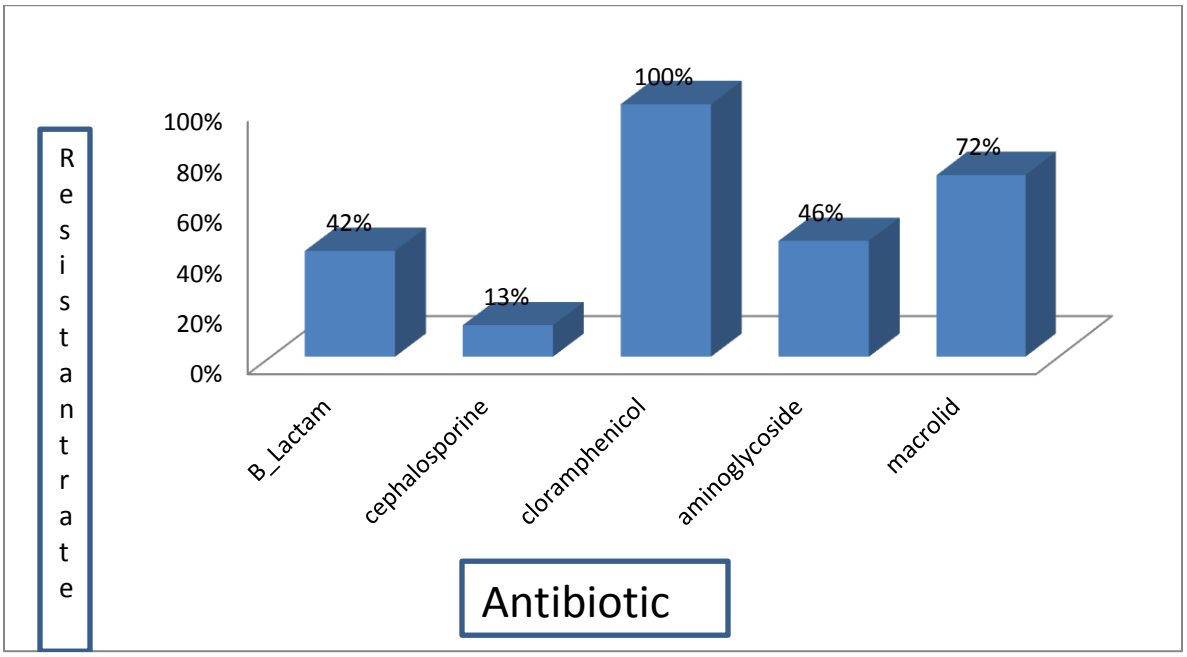

Figure (2) Antibiogram of *Streptococcus agalactiae* isolates

The results of current study shows that 42% of isolate are resistant to B-lactam antibiotic. Others studies like (Suzanne et al.,2011),(john et al., 2015) reported that Group B streptococci are susceptible to Penicillin. Penicillin remains the first choice to treat GBS infections, although strains with reduced susceptibility to this antimicrobial agent have been recently described. In addition, GBS resistance to alternative therapy, such as macrolides and lincosamides, has emerged in the last decades (Rosana et al,2012).

In present study, (13.5%) of GBS were resistant to cephalosporine antibiotics**.** (Hiroyuki *et al* 2012)found in his study that of all isolates were fully susceptible to cephalosporin.

However , Imepenem and meropenem , showed high effect on bacterial isolates where all isolates are sensitive to it. This may be due to its being new generation of Carbapenems that are now introduced in treatment in UTI and vaginal infection and this finding are identical with those obtained by Boroumand *et al.*, (2007) .

GBS isolates shows high resistance to aminoglycoside 46 %.(Alkhalidy 2013) in his study found that isolates were resistant to Gentamycin at rates 90%.

The first gentamicin high level resistant (HLR) GBS was described in France in 1990. The gene of the bifunctional aminoglycoside modifying enzyme Aac(6´)-Aph (2´´) that mediate resistance to gentamicin and all other aminoglycosides besides streptomycin as well as homologes to *aphA-3* and *aad-6* genes was identified. The aminoglycoside resistant determinants were carried by the transposon Tn*3706* located on the chromosome. Tn*3706* could be transposed from its chromosomal location onto the plasmid IP501, a conjugative multiple antibiotic resistance plasmid that can be transferred and stably maintained in a variety of Gram-positive genera, including GBS. The clinical impact of HLR aminoglycoside resistance is a loss of the synergistic bactericidal effect achieved in conjunction with cell wall active antibiotics as penicillin, which will compromise the effectiveness of therapy. The combination of a cell wall active antibiotic and an aminoglycoside has been shown to greatly enhance the killing of GBS *in vitro* (Granlund *et al*.,2010)

In Study done in Kuwait, (samar *et al*., 2012) a higher proportion of isolates (76.9 %) resistant to high-level kanamycin. It is uncertain whether this observation represents a new development in Kuwait or a new awareness of a previously existing problem. kanamycin-resistant

GBS isolates carried aph3 with nucleotide sequences that were 99% similar to aph3 found in E. faecium, E. faecalis, S. aureus and S. epidermidis, suggesting that GBS could have acquired aph3 from other Gram-positive bacteria. However, unlike kanamycin High-level aminoglycoside resistance in GBS is mostly due to enzymic inactivation of the antibiotic by N-acetyltransferases, O-adenyltransferases and O-phosphotransferases, encoded by aac, ant and aph, respectively.

Macrolide antibiotics, especially erythromycin, are important therapeutic agents for penicillin-allergic patients suffering from *S. agalactiae* infections The result that was obtained showed high resistance to Macrolide antibiotic (72%). Similar results in which its founds that rate of resistance to macrolides was 69 % (Facconi *et al*., 2010) in his study found that resistance rate to macrolides was 33%.

This rate of resistance was high can be compared to others reports like (Suzanne *et al*.,2011) whofound that rates of macrolides resistance relatively low in GBS isolates. This increase may be related to the increased use of macrolides in the hospital

Resistance against MLS_B antibiotics among *S. agalactiae* may be occurring through two mechanisms: through target site modification and through an active efflux pump. Target site modification is encoded by the erythromycin ribosome methylase (*erm*) genes [*ermB* and ermTR], conferring resistance to MLSB antibiotics. Phenotypic expression of

MLS_B resistance can be inducible ($iMLS_B$) or constitutive ($cMLS_B$). On the other hand, an active drug efflux system that functions via a transmembrane pump encoded by the*mefA* or *mefE* gene is responsible for macrolides and group B streptogramins resistance only. Expression of macrolide resistance in *S. agalactiae* isolates may be constitutive or inducible. When expression is constitutive who found that the isolates are resistant to erythromycin and clindamycin antibiotics. However, when the expression is inducible the isolates are only resistant to erythromycin(Mohammad *et al., 2010)* .

GBS isolates showed high resistance to Chloramphenicol (100%). Similar finding recorded in other studies like (Samar *et al*., 2012) .

In contrast, (45%) resistance to chloramphenicol was found in Iran. However, high prevalence of chloramphenicol resistance in *S. agalactiae* was also detected in Turkey (44. 2%) and Kuwait (30%). (*Mohammad et al., 2010)*. Chloramphenicol is rarely used in women especially in pregnant women because of its to(txicity and also its being the cause of anemia and gray baby (Alkhalidy , 2013). Resistance to this antibiotic is generally due to the synthesis of the enzyme chloramphenicol acetyltransferase, encoded by the gene *cat*, which inactivates the antibiotic by chemical conversion, losing the ability to bind to the ribosomes. In GBS, both chromosomally integrated through transposons and plasmid carried *cat* genes have been detected.

(*Streptococcus agalactiae* causing human infections: genetic diversity and capsular switching)

Extensive use of antibiotics in medicine and animal husbandry results in increased antibiotic resistance among bacterial populations. Several studies have suggested that antimicrobial use in animals causes the development of antibiotics resistance among pathogens in humans. (Beenu *et al*., 2012)

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