

ISOLATION OF STREPTOCOCCUS AGALACTIAE FROM WOMEN WITH UTERINE TUMORS**Hadeel T AL-Hadithi* , Haifa ALShaheen@ & Kudus E Omar#**

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Abstract

Streptococcus agalactiae was isolated from eight out of 42 cases of uterine tumors and endometrial hyperplasia from inpatients underwent total abdominal hysterectomy in Basrah Maternity and Child Hospital, due to irregular vaginal bleeding not responding to medical and hormonal treatment. Six isolates were from uterine tumors, and two from endometrial hyperplasia. Isolates were identified by being Gram positive, negative for catalase, giving positive CAMP test, ability to grow in 6.5% NaCl but not 40% Bile salts and resistance to Bacitracin.

In vitro studies were conducted to investigate virulence factors of isolated *S. agalactiae* by testing their ability to produce haemolysin, yellow pigmentation, capsule, resistance to tetracycline which is indicative for the existence of sialic acid (antiphagocytosis material). Measurement of the content of sialic acid and lipotechoic acid revealed differences between isolates.

All isolates, were able to adhere to plastic surfaces and formation of biofilms in both neutral (pH=7) and acid (pH=4.5) media. The present study has recorded for the first time, resistance of four isolates of *S. agalactiae* (50%) to Vancomycin. This finding is worrisome from the clinical point of view, as these isolates may become a potential source for transmission of Vancomycin resistance to other bacteria.

Introduction

Streptococcus agalactiae also known as Group B Streptococcus (GBS), is part of the normal flora of the human gastrointestinal tract, female urogenital tract and is the leading cause of bacterial infections in human newborns and immunocompromised adults emphasizing the opportunistic nature of the infection (Lerner, et al. 1977; Schuchat, 1999). GBS is increasingly recognized as pathogen in adult population, including the elderly, pregnant women, and diabetics (Gotoff, 2002).

For the establishment of a successful infection, *S. agalactiae* needs to adhere to epithelial surfaces, eventually penetrate them, and also protect itself against the immune system. For this purpose, *S. agalactiae* possesses several surface proteins that interact with proteins of the host extracellular matrix.

Polysaccharide antiphagocytic capsule has been identified to contribute to invasive GBS disease in human neonates (Edwards, et al. 1982; Takahashi, et al.1999; Lewis, et al.2007). Also GBS display beta-hemolysis (Forquin, et al. 2007) known for its ability to damage erythrocytes, lung epithelial cells (Doran, et al. 2002), and brain microvascular endothelial cells (Doran, et al. 2003). Besides, pigment is considered a virulence factor associated with the hemolysin/cytolysin (Liu, and Nizet, 2004; Liu, et al. 2004).

Correlation between elevated levels of these extracellular products and severity of infection has been a debate by many workers (Pollack, 1976; Durham, et al. 1981). However, Takahashi, et al.(1999) have indicated that strains in the putative high- virulence clone might have unique

surface properties; for instance, containing significantly increased sialic acid content, a property that might contribute to increased virulence.

The objective of this study is to examine strains of group B streptococci isolated from endometrial hyperplasia and uterine tumors, for the extent and level of virulence factors produced by each and for their susceptibilities to vancomycin.

Material and Methods

Isolates :- Eight isolates of *S. agalactiae* from uterine tumors (n=6) and endometrial hyperplasia (n=2) were obtained after total abdominal hysterectomy from women suffering from irregular bleeding during the period from 14 April 2004 to 15 October 2005 at Basrah Maternity and Child Hospital (Omar, 2006). Isolates were identified by being Gram positive cocci, negative for catalase, positive for CAMP test, capable of growing in 6.5% NaCl but not 40% bile salts, hydrolyzing arginin and gelatin and were resistant to bacitracin (Facklam, et al. 1991; Collee, et al. 1996; Kilian, 1998) Isolates were maintained in Todd Hewitt broth and tryptose blood agar. For testing, the isolates were grown on trypticase soy agar supplemented with 5% sheep blood at 37°C for 18 to 24hrs.

Detection of virulence factors

Demonstration of the capsule: - It was achieved by inoculating isolates on Congo red medium (Freeman, et al. 1979) then at 37°C for 24hrs. Existence and intensity of capsules were determined by the appearance of either highly viscous, transparent, black colonies or moderately viscous, dark pink colonies.

Detection of Sialic acid:- The method of Nagano, et al (1989) was adopted. Briefly, 4mg/ml of purified Tetracycline (Moffat, et al. 1986) was added to Todd Hewitt Glucose Broth, THGB (Omar, 2006); 2ml aliquots were distributed into

test tubes, then inoculated with lapful's of isolates and incubated anaerobically at 37°C for 24hrs. Appearance of turbidity (growth) was indicative of resistance to tetracycline owing to the presence of sialic acid.

Measurement of quantities of sialic acid and lipotechoic acid in the cell wall: The method of Terleckyj et al, (1974) was adopted. Isolates were inoculated onto, THGB and incubated at 37°C for 24hrs. A white thick precipitate was formed at the bottom of the tubes. Growth was monitored by measuring the absorbance in a Uvikon 810 spectrophotometer at 675 nm. The observed optical density was multiplied by 1,000 and converted to adjusted optical density units so that readings would agree with Beer's Law and be proportional to bacterial mass.

(Toennies and Gallant. 1949, cited in Neolon, T.J. and Mattingly, S.J. 1983). Turbidities were measured by dry-weight determinations of 10-ml samples of washed, stationary-phase cell suspensions.

Samples were dried at 84°C for 48h and desiccated over Ca₂SO₄ before weighing. It was ascertained that 1 adjusted optical density unit corresponded to 0.43µg of cellular dry weight per ml.

Pigment production: Colonies were inoculated onto THGB, the production of orange to red is a positive result (Gupta and Briski, 2004).

Blood haemolysis: The method of Finegold and Baron, (1986) was adopted. In brief isolates were cultured on Tryptic Blood agar supplemented with 7% sheep blood, incubated at 37°C for 24 hr. in an anaerobic jar. Formation zones surrounding colonies was reported positive result.

In Vitro adherence capability (Biofilm formation): The method of Christensen, (1985) was adopted with some modifications. Briefly, isolates were inoculated in THGB incubated at 37°C for 8 hrs (reaching up to the stationary phase), then cultures were centrifuged by

a cooling centrifuge at 5000x rpm. The precipitates were washed thrice with phosphate buffer solution (PBS) and put in 0.02 quantities in the wells of the 96-well microtitre plates (Duplicate plates were prepared to obtain replicates for each isolate). Tryptic soy broth was prepared and divided into two parts: one part was adjusted to be acidic (pH=4.5) and the second neutral (pH=7); the acidic part was added to one of the microtitre plate and the neutral part to the second one, then plates were incubated aerobically at 37C° for 24hrs. Wells were emptied, washed off five times with PBS, fixed with 25% formalin then stained with crystal violet. Degree of adherence was measured by Enzyme linked Immunosorbent Assay (ELISA) Reader at a wave length of 675nm.

Antibiotic Susceptibility to Vancomycin:

It was investigated by disc plate method (Mason, et al. 1996). Discs of vancomycin (30µg) supplied by Oxoid/UK were placed on Muller Hinton agar plates supplemented with 5% blood, each of which was seeded with one of the 8 isolates, grown overnight in Todd Hewitt broth, incubated at 37C° for 24hrs. according to existence of inhibition zone or not, results were recorded as sensitive or resistant.

Results

Although the 8 streptococcal isolates under study displayed typical positive results in the CAMP tests, but variation in the intensity of CAMP test was clearly obvious among isolates (Table I).

Determination of Virulence factors:

Demonstration of Capsule: The eight isolates of *S.agalactiae* have developed pink, moderately viscous colonies when grown on Congo red agar.

Resistance to Tetracycline: Isolates were capable of growing in (THGB) containing purified tetracycline, which reflect their resistance to tetracycline. Growth of isolates in this medium is also

indicative of the presence of sialic acid in their cell wall.

Estimation of quantities of Sialic acid and Lipotechoic acid in the cell wall of *S.agalactiae* isolates: Heavy growth with a heavy precipitate was remarkably observed (Plate 1) when isolates were grown in THGB, which indicates liberation of sialic acid and lipotechoic acid. Table II, illustrates the quantitative data of cell-associated material from these strains. These results, therefore, strongly suggest that high levels of these cell surface polymers directly correlate with the disease process and that they may play a role as potentially important virulence factors.

Reading of the optical density for precipitate produced by each isolate revealed that the highest value of which was demonstrated by two isolates from two cases of uterine tumors: UT2 and UT3.

Pigment production as correlated to haemolysis: The six isolates of *S.agalactiae* obtained from uterine tumors, gave rise to yellow colonies when grown in Islams medium. On the other hand when those isolates grown on THGB were left in the fridge at 4oC for 48hr. they displayed red coloration and were found tightly stuck to the inner surfaces of the tubes (Plate 2). These isolates have also produced β-haemolysis. In contrasts, isolates of *S.agalactiae* obtained from endometrial hyperplasia did not produce pigments and were non-haemolytic.

Adherence of *S.agalactiae* isolates In Vitro and biofilm formation: all the eight isolates of *S.agalactiae* under study were found capable to adhere to the wells of microtitre plates in either neutral or acid medium. Table III illustrates that intensity of biofilms produced was varied among isolates; the highest of which was revealed by UT2 and UT5 at acid and neutral pH respectively.

Resistance to antibiotics: Four isolates: UT1, UT3, UT4, and EH1 were found

susceptible to vancomycin, making up 50% resistance to vancomycin. This study reveals for the time, resistance of *S.agalactiae* isolates to vancomycin.

Discussion

Scarce information is available on the bacteriology of pelvic tumors. The average number of bacterial species isolated by Mikamo, et al. (1993) from inside the cervical cancers was 6.3., and *Streptococcus agalactiae* was among the predominant bacteria. Regardless of the presence of odor or treatment, VON Gruenigen, et al. (2000) have isolated aerobic and anaerobic bacteria equally from malodorous and non-malodorous gynecologic cancers and reported an average of five bacterial species but they could not assess the role of individual pathogens. Therefore, the question of whether a specific bacterium alone causes a cancer not answered. The present study has focused on virulence factors produced by *Streptococcus agalactiae* which may throw light on the role of this bacteria in such disturbances. GBS produce an orange carotenoid pigment, a unique feature that is useful in distinguishing GBS from other β -hemolytic streptococci (Tapsall, 1986). A link between pigment and hemolytic activity has also been described by Rose-Fraile, et al. (2006) and Forquin, et al. (2007) who also reported that level of beta-hemolytic activity correlates with the amount of the red pigment produced by the organism. Liu, et al. (2004) have indicated that β H/C is responsible for the characteristic zone of clearing around GBS colonies grown on blood agar plates and is capable of forming pores in a variety of eukaryotic cell membranes and that β -hemolysin /cytoysin (β H/C) encoded by *cylE* is the important virulence factor of GBS. Interestingly, *cylE* also contributed to enhanced survival within phagocytes (Texiera, et al 2001) that was attributed to the ability

of carotenoid to shield BS from oxidative damage. Deletion of *cylE* was found to result not only in the loss of β H/C activity, but also in the loss of a carotenoid pigment (Sperllberg, et al. 1999, 2000; Pritzlaff, et al. 2001). GBS generate a unique protein, the so-called CAMP factor, which interacts with the plasma membrane of red blood cells (PRCs) and other cell types (Hanson and Sorensen, 2003). In the present study, although all *Streptococcus agalactiae* isolates were positive for CAMP test (Table 2), but the two isolates: UT1 (gave weak CAMP reaction) and EH1 (gave strong CAMP reaction) were non haemolytic. This finding agrees with that of Sigga, et al. (2007) who reported heterogeneity of hemolysin expression displayed by a strain causing neonatal sepsis. Moreover, those two isolates (UT1&EH1) were non pigment producers, which might be attributed to qualitative variation in the composition of pigment produced in different strains of group B streptococci (Ring, et al.2000). However Gottschalk, et al. (2006) have indicated that nonhemolytic and non-pigmented phenotype may result owing to mutation of the genes.

An in Vitro study has clarified ability of GBS isolates from both sites for adherence in either acid or neutral condition with no significant difference. This finding contradicts with Tamura, et al. (1994) who reported that adherence was increased 6-to 20-fold at pH4.

Both hemolysin production and adherence of bacteria to host cells are presumed to be the initial step in and prerequisite for successful colonization promoting invasion of deeper tissues and ultimate dissemination of the bacteria to the bloodstream and multiple organ systems (Tamura, et al. 1994). Bekmann, et al. (2002) and Mikamo, et al. (2004), have identified microbial and host receptor-ligand interactions and reported that many streptococci express proteins

that bind specifically to proteins of the extracellular matrix (ECM) and/or serum. A critical factor contributing to GBS virulence is its surface capsular polysaccharide that forms the outermost layer of bacterial surface. The most extensively studied and widely appreciated virulence factor of GBS is its Sia-capped capsular polysaccharide (Lewis, et al.2007). The increased virulence results at least partially from the higher sialic acid content, which inhibits both opsonophagocytic killing and C5a production in the absence of type -specific antibody (Takahashi, et al. 1999). Also, LTAs mediates the attachment of pathogenic organisms to host cells (Wcken and Knox. 1975). They bind spontaneously to erythrocyte and epithelial cell membranes and have been suggested to play a central role in the pathogenesis of group A streptococcal infections (Beachy, et al. 1979; Ofek, et al 1977). The high level of LTA and sialic acid reported in the present study among uterine tumors isolates is, therefore indicative of their high degree of virulence. Nealon and Mattingly (1983) have indicated that strains from infants had significantly higher levels of LTA on their cells than did strains from asymptotically

colonized infants. Interestingly, both nonhaemolytic and nonpigmented isolates (UT1 and EH1) have displayed the least quantities of sialic acid and LTA.

Vancomycin resistant enterococci (VRE) have emerged as nosocomial pathogens in the past 10 years, causing epidemiological controversy (Song, et al.2008). Increased isolation of enterococci in the endometrium was found to be associated with use of cephalosporin prophylaxis, increased numbers of vaginal examinations and increased length of internal monitoring (Wallmer, et al. 1988). Considering the fact that GBS is part of the normal flora female urogenital tract, hence spread of antibiotic-resistance may have arisen both by clonal dissemination and by the horizontal transfer of resistance genes (Mellisa, et al. 2004; Gherardi, et al 2007). This might explain resistance of 4 out of 8 GBS isolates to vancomycin. Hence, emergence of vancomycin resistant GBS becomes of major concern. Therefore, a better understanding of factors associated with antibiotic resistance is needed to minimize group B streptococcus disease risks and to maximize effective chemoprophylaxis.

Table I: Variation in the intensity of CAMP test among isolates

Isolate No.	CAMP Test
Cases of Uterus Tumor (UT)	
UT 1	+
UT 2	+
UT 3	+++
UT 4	++
UT 5	+
UT 6	++++
Cases of endometrial Hyperplasia (EH)	
EH 1	+++
EH 2	+

Table II: Optical density (nm674) of precipitates produced by *S.agalactiae* isolates grown on Modified Todd Hewitt Glucose medium (MTHB)

Case No.	OD ($\mu\text{g/ml}$)	OD($\mu\text{g/ml}$) x1000	The value of each unit of OD=0.43 ($\mu\text{g/ml}$)of cell weigh in 1ml medium
Cases of Uterus Tumors (UT)			
UT 1	2.11	2110	907.3
UT 2	2.50	2500	1075
UT 3	2.38	2380	1023.4
UT 4	2.14	2140	920.2
UT 5	2.12	2120	911.6
UT 6	2.20	2200	946
Cases of Endometrial hyperplasia (EH)			
EH 1	2.18	2180	937.4
EH 2	2.26	2260	971.8

Table III: Intensity of biofilm produced by *S.agalactiae* isolates in neutral and acid pH

Isolate No.	Intensity of Adherence	
	Neutral medium (pH=7)	Acid medium (pH=4.5)
Uterus Tumor		
UT 1	0.241	0.197
UT 2	0.161	0.262
UT 3	0.242	0.139
UT 4	0.159	0.169
UT 5	0.266	0.199
UT 6	0.223	0.177
Endometrial Hyperplasia		
EH1	0.182	0.174
EH2	0.211	0.212

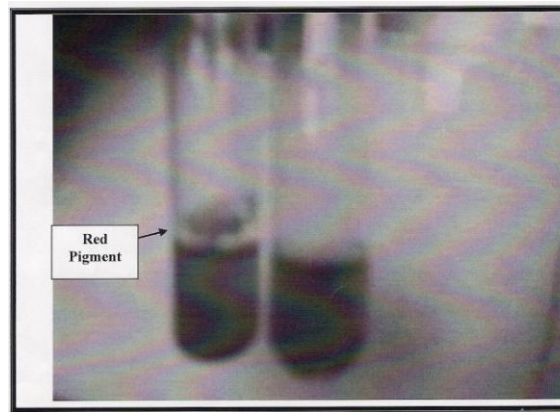


Plate (1) Adherence of Streptococcal colonies on the inner sides of the tubes with the formation of red colonies



Plate (2) Precipitates represent quantities of Sialic acid and Lipoteichoic acid in the cell wall of *S.agalactiae*

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