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SCREENING OF GROUP B *STREPTOCOCCUS AGALACTIAE* VIRULENCE FACTORS *(SCPB, SIP, CFB)* IN IRAQI PREGNANT WOMEN

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Abstract

We collected one hundred eighty nine from pregnant female this collection was from different age and different hospital after that we confirmed diagnosis by Vitek system and PCR amplification. We worked CAMP confirmed test to detected SGB after that we used genotyping identification to diagnosed V.F on all bacteria that appear positive to CAMP factor from result showed all bacteria that produce CAMP protein encoded by Cfb gene and consider major gene to encoded for CAMP factor, the other result used other V.F on this bacteria example C5a & sip gene.

Keyword: *Streptococcus*, GBS, CAMP test, *Cfb*, *C5a* and *sip* genes

Introduction

The first isolation streptococcus was from by bovine mastitis these discover had by Nocard and Mollereau in 1987 (Nocard and Mollereau, 1887). In human, Hare and Colebrook appear there are different in hemolytic between streptococci isolates that isolated from vaginal samples in parturient women and compares with or without puerperal fever. Found streptococci isolated from parturient women without puerperal fever consider same to these found in mastitis in cattle (Hare and Colebrook, 1934). Lancefield notice hemolytic streptococcus according to closer view called typing system provided hemolytic streptococci bearing her name (Lancefield 1934; Lancefield and Hare, 1935), and this depended on specific polysaccharides. There are specific polysaccharide in *S. agalactiae* fond by other study. There are four types of CPS were demonstrated and found to be strain specific; and supply system that consider the first typing system for GBS (Lancefield, 1934).

Pathogenicity

Neonatal bacterial disease causes by main cause consider *S. agalactiae* (Schuchat *et al*., 2006). UTI and skin and soft tissue this including in clinical widely of Group B Streptoccus disease consider broad in young adult (Farley and Strasbaugh, 2001). The other clinical presentation but n low frequent abd this include endocarditis and meningitis, this bacteria found related with considerably highest mortality and morbidity (Domingo *et al.,* 1997; Sambola *et al*., 2002). Found that the GBS could transmitted by vertical transition and could colonized from mother to their newborns and this occur in about 50%of birth. (Edwards and Nizet, 2011). This bacteria may by trans the disease by secondary route into uterus and then translocation by taught membrane by rupture membrane or translocation into birth canal via (Shet and Ferrieri, 2004). If the mother was high colonized mean female increase danger of colonized in newborn. (Regan *et al*., 1996, Shet and Ferrieri, 2004).

Virulence Factors (V.F) of GBS

The progress of GBS sickness this lead to that this bacteria continue in colonized and has the ability to invasive host by breakdown physical barrier. The V.F (virulence factor) in this bacteria are adhere factor which adhere to epithelial cell in host, the second and third V.F are endothelial and interference barrier and the interference intact innate immune clearance mechanisms (Martins, 2011).

CAMP Virulence Factor

The binding protein that have molecular mass almost 23.5 *Dalton* of *S. agalactiae* this term CAMP factor (Podbielski *et al.,* 1994). CAMP factor strong acting as beta toxin in staphylococcal sphingomylinase. The lethal property of this factor of cellular culture to cell rabbits and mice it might are o mammalian tissue (Jain *et al*., 2012). CAMP factor consider is an extra-cellular protein secreting via GBS, CAMP induced hemolytic phenomena. When grown on general blood agar plates next to a colony of *S. aureus* & *S. agalactiae* (Lang and palmer, 2003). This phenomena was first discovered in 1944 by Christie, Atkins and Munch-Petersen who known it the (CAMP reaction) (Christie *et al*., 1944). This reaction is found using to the conformed diagnosis of *S. agalactiae* that consider in clinical isolate (Ke *et al*., 2000). fund the hemolytic in zone develops in the neighboring region of the two streaks of *S. agalactiae* and *S. aureus* if CAMP factor present, this depended on the red cells are expose to both CAMP factor and sphingomyelinase (Donkor, 2007). Bernheimer were the first to intensively explore the nature and mechanism of the CAMP reply (Bernheimer *et al*., 1973). *S. agalactiae* these bacteria consider obligated parasite when invasion epitehelial and tissue that found in mammary glands, and eradication of individual from herds is, (Jain *et al*., 2012). There are stated Classification of this factor (CAMP factor) that consider as a virulence factor as showed by Koch's postulate still controversial since most authors have shown the mortality of rabbits were increase and mice after purify CAMP factor and injected with it. (Chuzeville *et al*., 2012), while the other appear cannot any infected could not determine any effect of the removal to the (CAMP factor that encoding to gene (*cfb*) on GBS pathogenicity (Hensler *et al*., 2008).

Consider *cfb* genetic gene is a universal in GBS strains, for that the factor (CAMP factor) identified via search *cfb* gene via PCR had been usually using to distinguish GBS bacteria from other streptococci (Chuzeville *et al*., 2012). Also appear the CAMP factor found in other G+ve bacteria belong to other streptococci including *Streptococcus*

pyogenes, *Streptococcus uberis* (Jiang *et al*., 1996), *Streptococcus porcinus*, *Streptococcus canis* and *Propionibacterium acnes*. (Hassan *et al*., 2000).

Surface Streptococcal C5a- Peptidase (*scp*B gene)

C5a peptidase consider protease found on streptococcus and also called surface serine protease because it local on surface, this protein act inactivation C5a during complement activation (Harris *et al*., 2003). Found that C5a peptidase secretion from of *S. agalactiae* and this enzyme breakdown human C5a other species, include mice (Bohnsack *et al*., 1993). Fund that thought streptococcal enzyme aid in virulence via interfering with N.R (neutrophil recruitment). Last search appear that promote bacterial invasive of epithelial cells via the C5a peptidase bind fibronectin (Fn) (Koroleva *et al*., 2002). There was alternative function of C5a peptidase, this occur genetically via genetic polymorphism, but no affected its ability to bind on fibronectin (Tamura *et al*., 2006). The study research show that the structure of C5a-peptidase is consist of component call C5a peptidase is consider key molecule in the complex interactions between host with streptococcus and this depended on hypothesis. First recognized in *S. pyogenes* was peptidase, and show the same enzyme that was found in *S. agalactiae,* and these two proteins called ScpA and ScpB (Koroleva *et al*., 2002).

Surface Immunogenic Protein (*sip gene*)

There are gene found in every serotype of GBS (Persson *et al*., 2008). This gene called *sip* gene, this gene encoded to *sip* protein and consider found in GBS bacterial surface also this protein secretion in addition found on bacterial surface (Rioux *et al*., 2001). Moreover, this sip gene consider high conservation with isolates from six different serotype these percentage almost 98%in identity (Springman *et al*., 2009).

Materials and Methods

Laboratory Prepared Media

Columbia Blood Agar Base :

Columbia blood agar(CBA) base media was prepared by adding 39g to one liter of distilled water. The medium Boiled until it is dissolve completely. After that Sterilizing via autoclaving for 15 minute at 121°C. Cooled to 50°C and 5% of sterile defibrinated blood was added (Alia *et al*., 2011).

CHROM agar Strep B Media :

Amount of 44.7g of powder base Dispersed slowly in one liter of sterile water, 2g of supplement S1 was added into slurry. The agar Sterilized until it is solubilized. Autoclave was used at 121°C during 15 min. Cooling at 45/50°C keeping on stirring. In a transparent vessel, 250 mg of S2 supplement was suspended in 10ml of distilled water. After that, S2 supplement was placed under tension with a magnetic stirring until S2 is solubilized. Then 10ml of S2 preparation had add into (Base and S1) slurry and cooled at 45/50°C while mixing. Finally, the agar was swirled gently to homogenize and Poured into sterile Petri dishes. (Poisson *et al*., 2010).

Preparation of Solutions

Preparation of Lysozyme

Amount of 10mg of lysozyme powder was added to 1ml of deionized water to obtain stock solution as administered in Wizard genomic DNA purification kit.

Preparation of 50mM EDTA

The required concentration was obtained by adding the13.600 mg of EDTA to 1ml of water and dispersed by vortexing.

Preparation of RNase A

Amount of 10mg of RNase A powder was added to 1ml of deionized water to obtain stock solution as administered in brucher of kit.

Sample Collection

One-Hundred eighty nine swab were taken from the mucus of vaginal tissues of pregnant women at 36-40 weeks of gestation during 20th October 2013 to 22th February 2014 from Ibn al-Balady Hospital, Central Public Health Laboratory, Kamal al-Sammaraie Hospital. After that, transported swabs to the lab and cultured via used Amies transport media.

Bacterial Isolation

Every swab had been culturing primary on CAB via gently streaking on sterile prepared plates and then incubate at 37°C for 24-48 hours. After that, a typical growth was obtain.

After that, colonies that obtain from primary cultured, acted for it sub-cultured on CHROM strep B agar, this media consider a selective media for GBS isolation. We obtain blue and pink colonies. The blue colonies which belong to anther bacteria were neglected while the pink to moave colonies were sub- cultured into the same condition to get a single colony that was pure.

Identification Tests

Catalase Test

To differential between *Streptococcus* species from *Staphylococcus*, Catalase test was performed (the mechanism of this test achievement that the catalase enzyme acted to breakdown H_2O_2 into H_2O and O_2). This test was performed by Placing few colonies from growth on culture and put in the slid and then we put few drop of H_2O_2 and mixed ,the result consider positive if there is bubbles these test consider positive and these evidence GBS bacteria and if there is no bubbles these result consider negative. (Evans *et al*., 2009).

CAMP Test

This test used to differential between *S. agalactiae* and *S. pyogens*, the first step we straight *S. aureus* in straight line on blood agar plate after that we leave 2-3 cm and we straight the unknown bacteria by cross shape then incubated these plate at 37C for 18-24h. If the result was positive will formation arrowhead hemolytic between the Group B *Streptococcus* and junction of growth of *S. aureus*. If the tested isolate is not Group *B Streptococcus* this meaning there is no appear “arrowhead” hemolysis. (Lang *et al*., 2003).

PCR Amplification Procedure

All GBS isolates contain for three virulence factor genes *scp*B, *cfb* and *sip* which responsible for production of C5a peptidase, CAMP protein and Surface immunogenic protein respectively were screened. Table (1) represent the PCR mixture for each reaction in the current study. PCR condition were optimized by repeated changing temperatures ranged (47 to 61 °C) and number of cycle (30 to 35) as mention in table (2). generally steps started with initial denaturation step 96°C for 5 minutes followed by repeated cycles which consist from denaturation step ranged from 94°C to 96°C, annealing step depend on type of primer then elongation step mostly at 72°C followed by last extension step (usually at 72°C) (Joshi and Deshpande, 2011).

Table 1 : Solution mixture used for genes amplification procedure. The final volume was 25 µl for each PCR tube.

Target gene	Template DNA (µl)	F+R Primers (10 pmol/ml) (µl)	GoTaq®Green Master Mix (1x) (µl)	Nuclease free water (µl)
Cfb	5	1.5	12.5	6.5
***scp*B**	6	1.5	12.5	5
Sip	5	1.5	12.5	6

Table 2 : PCR primers sequences for target genes used in experiments.

Target Gene	Primers (5' to 3')	Product	Amplicon (bp)	Reference
*scp*B	**F:** ACAACGGAAGGCGCTACTC	C5a peptidase protein	255	Dmitriev *et al.*, 2004
	R: ACCTGGTGTTTGACCTGAA			
Cfb	**F:** TTTCACCAGCTGTATTAGA	CAMP protein	154	Bakhtiari *et al.*, 2012
	R: GTTCCCTGAACATTATCTT			
Sip	**F:** TGAAAATGCAGGGCTCCAACCTCA	Surface immunogenic protein	293	Castellano-Filho *et al.*, 2010
	R: GATCTGGCATTGCATTCCAAGTAT			

*** Manufactured by alpha, USA.**

Result and Discussion

Group B Streptococci Isolation

We were collected 189 swab from pregnant female this collected was during gestation period 38-46 week and were collected from different age that including 18-46 year .this sample collected from different hospital included (Ibn al-Balady Hospital, Kamal al-Sammaraie Hospital and Central Public Health Laboratory).the collected swab had transfer swabs by Amis transported medium and give to lab. Specific for microbiology and after that diagnosis by methods of culture then VITEK system and finally by PCR amplification. *S. agalactiae* was spread through pregnant female which belong Iraq Iraqi and the ratio was 24.3%. Group B *Streptococcus* almost 46 collected out study and diagnosis worked confirmed via different many tests before use it. This studies appeared that the maximum rate of group g streptococcus that infected female had thirty five strain and this infected take place between 35-37 year of female m, on other hand the infection was one sample 2.1 in pregnant female occurrence between (18-24) years age pregnant female as mention in table (3.9). Also, fund pregnant female that between 25-46 year age middle rate in infection and this rate almost (6.52%) and seven (15.2%) strains respectively as mention in scheme (3.9).

Phenotype Identification

Achievement CAMP confirmed Test

All of blood sample culture and show positive in CAMP test (Christie *et al.*, 1944). Fund that CAMP reaction test the CAMP reaction previous use in common to the confirmed diagnosis of *S. agalactiae* in isolate that get from hospital (Chuzeville *et al.*, 2012). Show that each isolate that positive in culture also consider positive in CAMP test and this result gave highest percentage almost thirteen that consider one handed percentage of typical diagnosis because the *cfb* gene, which is gene that main coding for CAMP, that consider factor is represent housekeeping gene in *S. agalactiae* (Krishnaveni *et al.*, 2014). In our result showed that the positive isolates consider positive by an "arrowhead" hemolytic formation between line of the *S.* aueus & *S. agalactiae* which was grow in blood agar medium Krishnaveni *et al.*, (2014) who appear the first step of the reaction by distinct half-moon area of hemolysin. There are material called sphingomyelin fund in RBCs of sheep consider change this material into ceramide and this change by enzyme sphingomyelinase that secreted from *S. aureus.* After that fund of the CAMP factor link to pretreated cell membrane and by this mechanism lead to lyses of cell.

Screening of Virulence Factor Genes

All isolates that examined when they acted screened to obtain diffusible extracellular protein (CAMP factor), that produced from GBS and this protein encoded by *cfb* gene(Bohnsack *et al.*, 2000). These gene consider major gene to encoded for CAMP factor and this factor fund in every GBS isolates and can developed identified by PCR assay (Ke *et al.*, 2000). The result in our study showed to establishment fund this factor we used conventional PCR assay and we used primer specific for GBS and this primer recognized *cfb* gene that consider found in all GBS isolates. CAMP factor genes are describe to be widely spread through streptococci, and found in sero-groups A, B, C, G, M,P, R and U (Gase *et al.*, 1999). Our result appear 45 (97.8%) of identification GBS isolates were contain *cfb* gene and this segment within this bacteria ,this result agreement with which Chuzeville *et al.* (2012).

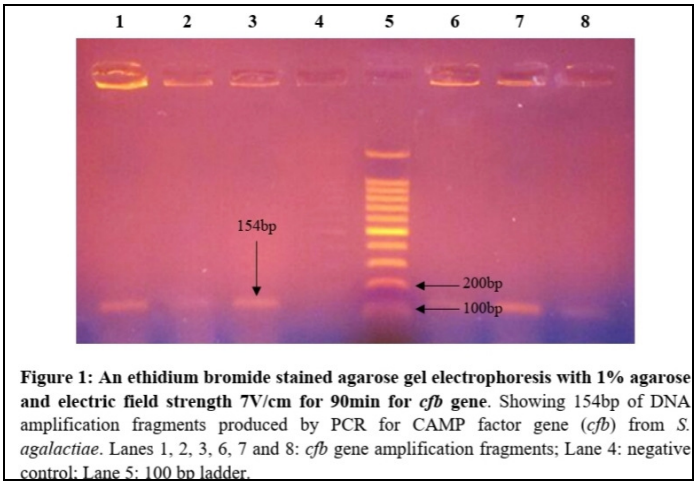

Figure 1: An ethidium bromide stained agarose gel electrophoresis with 1% agarose and electric field strength 7V/cm for 90min for *cfb* gene. Showing 154bp of DNA amplification fragments produced by PCR for CAMP factor gene (*cfb*) from *S. agalactiae*. Lanes 1, 2, 3, 6, 7 and 8: *cfb* gene amplification fragments; Lane 4: negative control; Lane 5: 100 bp ladder.

One from all examined isolates that was consider V40 consider (–ve) for *cfb* gene screening in this study .the PCR consider system that has many factor influence on it, these factor may be optimal extraction and DNA amount and purity (Mumy and Findlay, 2004; Alm *et al.*, 2000). In addition the 30 isolates that show positive in blood culture procedure consider half moon forming hemolytic zone on plate that contain sheep blood agar these back into influence of R-lyses of *S. auerus* and evidence that these isolates have classical CAMP phenomenon. The *Cfb-based* diagnosis is depended in detection GBS because consider tool to infected of this gene consider sensitive and reliable for these bacteria (Hassan *et al.*, 2000). The CAMP also found in other bacteria called *S. uberis* and this gene expression by other gene called *cfu gene* (Khan *et al.*, 2003).

Other studies appear there are second gene represent virulence gene found in this bacteria these gene called *scpB* gene and our result showed there are 45 isolates contain these

gene. Some of *S. aglacticae* has this gene (Chmouryguina *et al*., 1996; Herbert *et al*., 2005).

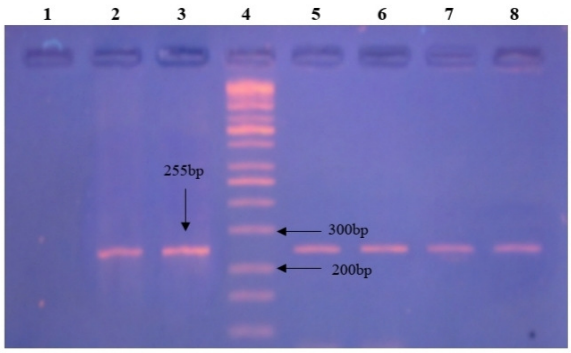

Figure 2: An ethidium bromide stained agarose gel electrophoresis with 1% agarose and electric field strength 7V/cm for 90min for *scp*B gene. Showing 255bp of DNA amplification fragments produced by PCR for C5a peptidase gene (*scp*B) from *S. agalactiae*. Lane 1: negative control; Lanes 2, 3, 5, 6, 7 and 8: *scp*B gene amplification fragments; Lane 4: 100 bp ladder.

There are similar result had fund to our result that indicated a positive result of *scpB* based assay (Slotved *et al*., 2007). Screening to *scpB* gene it was found only in 22.2% isolates that belong bovine isolates (Dmitriev *et al*., 2004), whereas Krishnaveni *et al*. (2014) found just 9% of bovine isolates had the *scpB* gene. The result appear contain just one (2.2%) of GBS detected isolates which it is V45 was negative in *scp*B by screening. Other scientist used PCR to detected *scpB* gene and if *scpB* gene found this mean the streptococcus consider GBS because this gene *scpB* sequence different from other strep. and this gene found just in GBS and size it almost 51-bp deletion, starting from nucleotide 3424. (Ke *et al*., 2000). In our result show *sip* gene consider high significant because these gene found in 45 isolates in our sample ,these rate existed that this bacteria could expression to sip protein and this protein consider surface immunogenic protein and surrounded on surface of this bacteria (Brodeur *et al*., 2000).

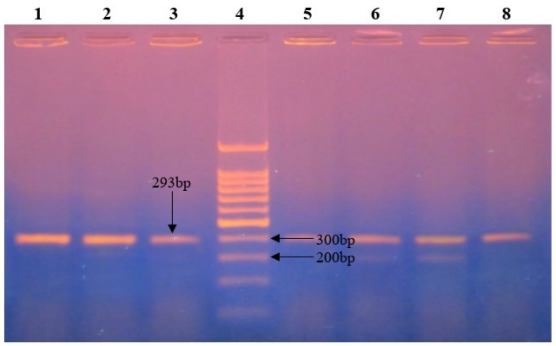

Figure 3: An ethidium bromide stained agarose gel electrophoresis with 1% agarose and electric field strength 7V/cm for 90min for *sip* gene. Showing 293bp of DNA amplification fragments produced by PCR for surface immunogenic protein gene (*sip*) from *S. agalactiae*. Lane 1, 2, 3, 5, 6, 7 and 8: *sip* gene amplification fragments; Lane 4: 100 bp ladder.

These studies compares result with other reported and showed high rate similar of *sip* harboring strains (Martin *et al*., 2002; Bergseng *et al*., 2007). Found just one (V13) of detected isolates (2.2%) were negative, The other we re-cultured to confirm and we worked PCR on these isolates and the result was positive and this evidence that *sip* found then the result was positive these evidence that *sip* gene found it but phenotypic negative and these evidence that this strain consider GBS strains and phenotypic consider false negative (Bergseng *et al*., 2007).

Table 3 : Rate of existence of virulence factors genes in all screened isolates.

Screened genes	Total screened isolates	Positive isolates	Negative isolates	Rate of existence
***Cfb* gene**	46	45/46	1/46	97.8%
***C5a* gene**	46	45/46	1/46	97.8%
***Sip* gene**	46	45/46	1/46	97.8%

Conclusion

Most of vaginal isolates are virulent since it contain combination of three-virulence genes *cfb*, *sip* and *scp* B that is encode for three virulence factors that colonize and invade the epithelial of vaginal tract.

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