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Comparative study for detection of group B Streptococci (GBS) by bacteriological culture and molecular methods

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Abstract

Group B *Streptococci* (GBS) is an important cause of invasive infection in newborn infants, in women around the time of childbirth and in older individuals with underlying chronic illnesses. Although GBS has the capacity to produce life threatening infection in susceptible hosts. One-Hundred eighty nine swabs were taken from the mucus of the vaginal tissues of pregnant women at 36-40 weeks of gestation during October 2013 to February 2014 from Ibn-Al-Balady Hospital, Central Health Laboratory and Kamal-Al-Samaraie Hospital in Baghdad, Iraq. The isolates initially identified phenotypically by blood agar culture media and chromogenic culture media, further identification performed by VITEK 2 system. Molecular methods was performed by using 16s rRNA gene based PCR. A comparative analysis were performed between the three phenotypic detection methods and molecular method were best tool for *S. agalactiae* detection, where in which 46 isolates were identified while 37, 36 and 30 isolates were detected by VITEK 2 system, chromogenic agar and blood agar respectively. VITEK Antibiotic Susceptibility Test for different antibiotics performed to all detected isolates, 45(23.8%) GBS were detected by 16S rRNA gene based PCR, The advantage of this method is that results can obtain within 1 day as compared to traditional microbiological testing. In addition, 16S rRNA gene is accurate rapid tool for identification.

Keywords: Group B Streptococci, Bacteriological culture, Molecular methods

Introduction

Lancefield group B *Streptococci* (GBS) or *S. agalactiae* is a gram-positive encapsulated bacterium exhibiting. (Makarova *et al.,* 2006). *S. agalactiae* is a species in the genus of *Streptococcus*. The members of this genus are facultative anaerobic and their metabolism is mainly fermentative (Whiley and Hardie, 2009). This bacterium is 0.6-1.2µm in diameter, non-motile, non-spores forming (Schuchat, 1998). *S. agalactiae* is a bacterium that colonizes the gastrointestinal and genitourinary tracts of healthy individuals without any symptoms of disease (Verani *et al*., 2010). Nevertheless, this bacterium can cause life-threating invasive diseases in pregnant women, neonates or non-pregnant adults. Among non-pregnant adults, GBS disease typically occurs among older or immunocompromised persons (Otaguiri *et al*., 2013). GBS is the main causative agent of invasive neonatal bacterial disease (Schuchat *et al*., 2006). The clinical spectrum of GBS disease in adults is broad, including more frequently bacteremia with or without sepsis, skin and soft tissue, osteoarticular and urinary tract infections (Farley and Strasbaugh, 2001). Detection of GBS on sheep-blood agar showed that it is form a unique ring-like zone of ß-hemolysis around the colony. However, a small proportion of GBS (3-11%) can be non-hemolytic. The CAMP test (named after Christie, Atkins and Munch-Petersen) used for the presumptive identification of GBS, yielding positive results for up to 98% of GBS isolates (Koneman, 2006) it is because GBS produce an extracellular protein called CAMP factor. Polymerase chain reaction (PCR)-based assays offer promising tools for sensitive, specific and rapid detection of GBS directly from clinical specimens at the time of delivery, by passing the need for lengthy culture of GBS. PCR assays for identification of GBS have developed by targeting a variety of genetic targets, including genes encoding C protein (Mawn *et al*., 1993), the 16S rRNA (Bäckman *et al*., 1999; Wang *et al*., 1999), and the 16S–23S spacer region (Hall *et al*., 1995). This PCR assay allows the detection of as little as one genome copy of GBS. In addition to its excellent performance with purified genomic DNA samples, the PCR assay achieves almost the same sensitivity as standard culture methods when

vaginal and/or anal specimens from pregnant women tested for GBS colonization (Bergeron *et al*., 2000).

Materials and Methods

One-Hundred eighty nine swabs were taken from the mucus of the vaginal tissues of pregnant women at 36-40 weeks of gestation during October 2013 to February 2014 from Ibn-Albalady Hospital, Central Health Laboratory and Kamal-Al-Samaraie Hospital in Baghdad, Iraq. Then, swabs transported by Amies transport media to the laboratory for further study.

Biological culture detection: Each swab cultured firstly on Columbia blood agar by gently streaking on sterile prepared plates and incubated at 37°C for 24-48 hours. Then, a significant growth obtained for microscopic examination, catalase test and CAMP test in which a known hemolytic strain of *Staphylococcus aureus* streaked in a straight line across the center of the sheep blood agar plates. The tested isolates of GBS are streaked in a straight-line (2-3cm in length) perpendicular to *S. aureus* streaking but without touching it. The plates incubated at 37°C for 18-24hrs. A positive test of CAMP factor appears as "arrowhead" hemolysis between the junction of growth of *S. aureus* and Group B *Streptococcus*. There is no enhanced or "arrowhead" hemolysis if the tested isolates are not Group *B Streptococcus*. (Lang *et al*., 2007). All the swabs cultured on CHROMagar Strep B media, a selective media for GBS isolation. Pink to mauve, blue and colorless colonies obtained after incubation aerobically at 37°C for 24-48 hours. The pink to moave colonies expected GBS as referred in the media leaflet sub-cultured in the same previous condition to get pure single colonies while blue and white colonies, which belong to another bacterial species, neglected. All the positive isolates confirmed GBS by Vitek system assay.

Molecular Detection: The genetic detection method was performed by using 16s rRNA gene based PCR which it is the most common housekeeping genetic marker for a number of reasons; include, its presence in almost all bacteria, often it is existence as a multi-gene family, or operons. The function of 16S rRNA gene over time has not changed, suggesting that random sequence changes are the most accurate measure of time (Janda and Abbott, 2007).

This housekeeping gene used for genotypic detection of GBS by PCR technique. DNA extracted from swabs prepared by using Wizard® Genomic DNA Purification Kit. Specific primer used for target gene manufactured by Promega, USA; Forward sequence GCCTCATAGCGGGGATAAC and reverse sequence ACGTTCTTCTCTAACAACA with 328bp expected amplicon (Mian *et al*., 2009). The solution mixture used for gene amplification procedure by PCR with final volume 25µl were template DNA 5µl, primer after dilution 1.5µl, master mix (1x) (GoTaq®Green) 12.5µl and nuclease free water 4.5µl. In addition, reaction conditions were initial denaturation temperature 96°C for 5min, denaturation temperature 96°C for 5min, annealing temperature 55°C, elongation temperature 72°C for 1min and final extension 72°C for 7min. The amplified PCR products screened by horizontal agarose gel electrophoresis. All the procedures followed according to the instructions of manufacturer companies.

Results and Discussion

The prevalence of *S. agalactiae* among pregnant women was 24.3%. Forty-six *S. agalactiae* isolates detected of all swabs and identified. The study showed that the highest percentage of GBS-infected women was between 35 to 37 years old with 35 isolates (76.08%) of all detected isolates, while, the lowest incidence of infection was between 18 to 24 year's old of pregnant women with one specimen (2.17%). As well as, the pregnant women between 25 to 35 and 37 to 46 years old displayed a moderate rate of infection reached three (6.52%) and seven (15.2%) isolates respectively as shown in Table (1).

Table (1): Percentage of infection among forty-six infected women according to age.

Age	(18-24) years	(25-45) years	(35-37) years	(38-46) years
Infected women	1	3	35	7
Percentage	2.17%	6.52%	76.08%	15.20%

Blood agar based diagnosis: All One hundred and eighty nine vaginal clinical samples were cultured on Columbia blood agar with 5% sheep blood. The obtained colonies of group B Streptococci tend to be larger and often have less pronounced zones of beta-hemolysis than do other beta-hemolytic strains (Engelkirk and Duben-Engelkirk, 2008). The study showed that *S. agalactiae* in culture is typically gray to whitish-gray in color, which is similar to the result of (Smyth and McNamee, 2008). Study showed that 30 (15.9%) of total vaginal specimens were positive using blood agar culture, as shown in Table (2). All positive ß-hemolytic strains tested by catalase test as mentioned in (Evans *et al*., 2009). All tested strains microscopically examined Gram-positive, short chains or diplococci were catalase- negative and positive CAMP factor (Christie *et al*., 1944). The

CAMP reaction used since it is widely used for the presumptive identification of *S. agalactiae* in clinical isolates (Chuzeville *et al*., 2012). Catalase and CAMP tests indicate a positive confirmation result of examined GBS isolates, in addition, Gram- positive, non-hemolytic cocci, and negative CAMP factor isolates were considered negative result also catalase positive cocci.

Chromogenic agar diagnosis: All vaginal swabs cultured on CHROMagar Strep B for detection and differentiation. GBS detected in 36 isolates (19.0%) of the 189 investigated vaginal specimens. All of the culture positive isolates were positive CAMP test, which is, gave high percentage reached 30(100%) of particular identification since *cfb* gene, which is coded for CAMP, factor is housekeeping gene in *S. agalactiae* (Krishnaveni *et al*., 2014). The positive isolates indicated by the formation of an "arrowhead" of hemolysis between the lines of the *S. aureus* and *S. agalactiae* growth on sheep blood agar. The distinct half-moon like area of hemolysis were explained by (Krishnaveni *et al*., 2014*)* who suggested that in the first step of the reaction, sphingomyelin in the sheep red blood cell membranes is converted to ceramide by sphingomyelinase from *S. aureus*. CAMP factor then binds to the pretreated cell membranes, leading to hemolysis of the cell. Christie, Fehrenbach and co-workers reported that CAMP factor might cause lysis of red blood cells that contain at least 45-mol percentage of sphingomyelin in the cell membrane (Barton *et al*., 1973; Bernheimer *et al*., 1979*)*. While it disagreed with the result (Krishnaveni *et al.*, 2014) since the phenotypic property was not demonstrable also in one isolate out of 15. May be due to lack of expression of the gene, or could be due to the absence of complete open reading frame (ORF) (Krishnaveni *et al.*, 2014). The isolated GBS in both biological culture methods confirmed positive by Vitek system assay.

Genetic detection of *S. agalactiae*: Molecular GBS identification performed by 16S rRNA gene based PCR. The entire one hundred and eighty nine collected specimens tested by PCR to ensure accuracy and to find the most suitable method for GBS detection. In vaginal specimens, 45(23.8%) GBS were detected by 16S rRNA gene based PCR as agreed with (Mian *et al*., 2009). Sensitivity of PCR in diagnosis of *S. agalactiae* can improve by choice of a suitable extraction method (Kim *et al*., 2001; Al-Soud *et al*., 2000). The advantage of this method is that results can obtain within 1 day as compared to traditional microbiological testing (Nilsson *et al*., 2003). In addition, previous work on other bacteria has indicated that 16S rRNA gene is accurate rapid tool for identification (Nilsson *et al*., 2003; Sacchi *et al*., 2002).

Comparative analysis of GBS detection methods: The prevalence of colonization among the 189 pregnant women was very high since its reach to 24.3%.

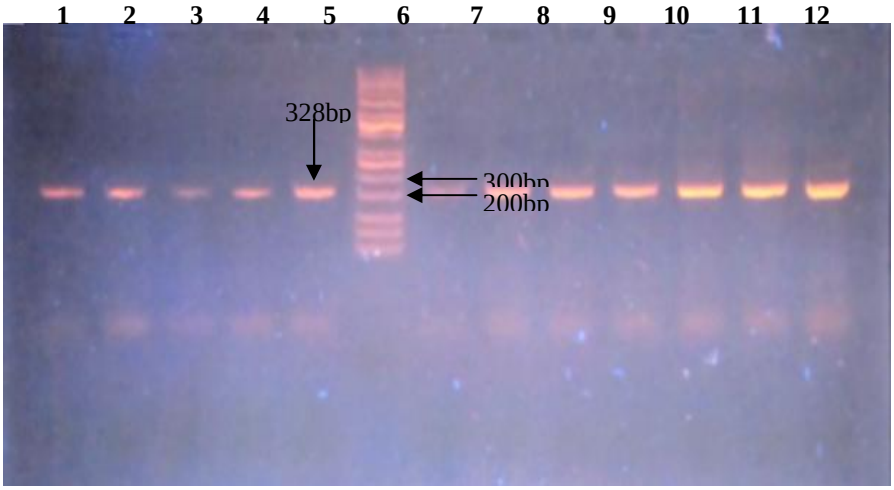

Figure (1):. An ethidium bromide stained agarose gel electrophoresis with 1% agarose and electric field strength 7V/cm for 90min for 16S rRNA gene. Showing 328bp of DNA amplification fragments produced by PCR for 16S ribosomal RNA gene from *S. agalactiae*. Lanes 1, 2, 3, 5, 7, 8, 9, 10, 11, 12 and 13: 16S rRNA gene amplification fragments; Lane 6: 100bp ladder.

Table (2): Comparative analysis of detection methods.

Detection	Method	Positive	Sensitivity/ Specificity	Cost	Result time
Blood agar culture	Columbia blood agar	30	65.2/100	Low	72hrs.
Chromogenic agar culture	CHROM agar StrepB	36	78.3/100	Moderate	24-48hrs.
Vitek diagnosis	Vitek kit	37	80.4/100	Moderate	24hrs.
PCR	16S rRNA	45	97.8/100	High	3.5hrs.

As PCR technique compared with the conventional culture method, GBS colonization was detected earlier (after 3.5hrs. for PCR versus 72 and 24hrs. for blood agar and chromogenic agar culture method respectively, and 24hrs. for Vitek diagnosis) and with a significantly higher sensitivity (97.8 versus 65.2%, 78.3% and 80.4%) by PCR method than culture methods and Vitek test. From a total of 46 GBS-positive specimens, 16 (34.8%) blood agar culture, 10(21.7%) chromogenic agar culture and 9(19.6%) Vitek diagnosed false-negative results, which were PCR positive, because of the high specificity of primer evaluated in this study; also, elimination of false-positive results showed in (Kemp *et al*., 2000). A specific primer as 16S rRNA gene used for the detection of GBS in swabs. The primer used for each swab to ensure the accuracy of PCR to exclude technique negative results (Bergeron *et al*., 2000).

All the methods used in this study showed specificity 100% for detection of GBS. Blood agar culture method showed a sensitivity of 65.2%, whereas chromogenic agar culture method showed 78.3% sensitivity for detection of GBS, while Vitek diagnosis method was 80.4% sensitivity. The PCR specific gene detection method was the more accurate technique with a sensitivity of 97.8%. The time that required for obtaining the results was 3.5 hrs for the PCR technique, whereas 72hrs. for the blood culture result. At least 24hrs. for chromogenic culture result are required and 24hrs. for Vitek diagnosis. However, the PCR technique takes more handwork than the bacterial culture with higher cost. The discrepancy between the number of positive results by PCR technique and culture might explained by several factors.

1. By analyzing the culture, usually only beta-hemolytic colonies selected as suspect and further examined. However, the frequency of strains are not hemolytic is estimated to be 2 to 5% (Savini *et al*., 2013).
2. The stability of *S. agalactiae* in vaginal swabs during transport may be a limiting factor for the culture method. In addition, the time between obtaining specimens and preparation of vaginal swabs in the microbiology laboratory varied between 30min. and 4 hrs. The above-mentioned advantage of PCR over culturing might be even more evident if larger transport distances or a delayed transport occurred.
3. Another possibility might be the fact that bacteria which have killed by antibiotic drugs and which cannot be grown by culture were still detectable by PCR (Rüssmann *et al*., 2001). This advantage of the PCR technique for the detection of microorganisms from antibiotic-treated patients also underlined in a recent study with clinical samples obtained from cystic fibrosis patients (Hraoui, *et al*., 2012).

Moreover, the data showed that one vaginal specimen (V4) which was GBS positive by blood culture and Vitek but GBS negative by chromogenic media culture. Furthermore, the study showed that 16 (34.8%) was negative result in cultures methods and converted to positive result by PCR detection. Most previous studies (Jordan *et al*., 2005; Liu *et al*., 2005) focused on confirmation of culture-positive results by PCR methods, with less attention to the alteration of negative cultures to positive results. (Matsuda *et al*., 2011) also showed that 10% of negative cultures contained bacteria, highlighting the clinical need for a method able to detect those organisms that cannot be detected with culture methods. (Fontana *et al*., 2005) showed that many factors suspected of contributing to false negative culture results include insufficient blood sample inoculum, empirical or long-term antibiotic use prior to diagnosis, and infection due to fastidious organisms.

Only one vaginal specimen was positive GBS in the standard culture methods and Vitek diagnosis but negative by PCR for the targeted gene. A possible explanation might be that insufficient amounts of DNA can lead to the failure of PCR to detect bacteria, as recently reported in similar study (Rüssmann *et al*., 2001). In fact, in this single case, a very low number of bacteria might be the reason for the failure of PCR to detect GBS.

Moreover, the limit of detection of bacteria by PCR corresponds to that of other microscopic methods and can be estimated to be 10^3 to 10^4 CFU/ml of tested sample as explained in (Artz *et al*., 2003). Similarly, (da Cunha Gonçalves-de-Albuquerque *et al*., 2014) showed that with any molecular diagnostic method, the potential for false-negative results is of concern when analyzing clinical samples. A false-negative result could be due to the presence of PCR inhibitors, poor target DNA recovery during extraction, degradation of target DNA before amplification, errors in setting up a reaction or degraded reagents (De Zoysa *et al*., 2012).

Conclusions

The study evaluated the PCR technique for the detection of GBS in vaginal specimens as a rapid, highly specific and sensitive using 16S rRNA gene, which corresponded with (Bergeron *et al*., 2000) (Picard and Bergeron, 2004). The development of PCR based methods provides a promising option for the rapid identification of bacteria. With this method, identification of bacterial pathogens can made in hours, rather than days required for biological culture methods. PCR can also improve the level of detection due to its high sensitivity. Theoretically, only a few cells of pathogen are necessary to yield a positive diagnosis (Phuektes *et al*., 2001).

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