

Authors Guidelines:

Electronic Proof Correction Using Acrobat Reader

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X). The latest version of Acrobat Reader can be downloaded for free at: <http://get.adobe.com/reader/>. Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



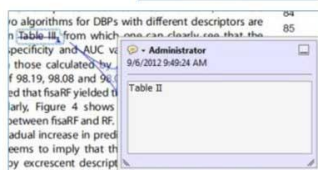
1. Replacement Text Tool — For replacing text.

Strikes a line through text and opens up a replacement text box.



How to use it:

1. Highlight a word or sentence
2. Select "Replace Selected Text" from the Text Edits fly down button
3. Type replacement text in blue box



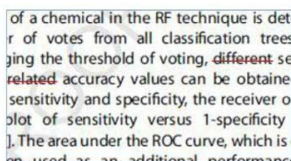
2. Cross-out Text Tool — For deleting text.

Strikes a red line through selected text.



How to use it:

1. Highlight a word or sentence
2. Select "Cross Out Text for Deletion" from the Text Edits fly down button



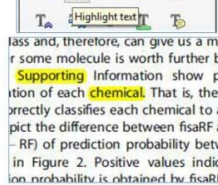
3. Highlight Tool — For highlighting a selection to be changed to bold or italic.

Highlights text in yellow and opens up a text box.



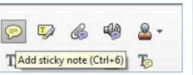
How to use it:

1. Highlight desired text
2. Select "Add Note To Selected Text" from the Text Edits fly down button
3. Type a note detailing required change in the yellow box



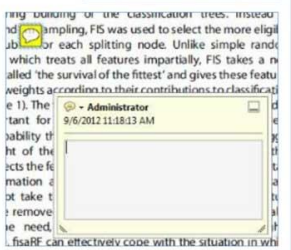
4. Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.



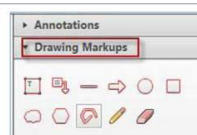
How to use it:

1. Select the Sticky Note icon from the commenting toolbar
2. Click where the yellow speech bubble symbol needs to appear and a yellow text box will appear
3. Type comment into the yellow text box



5. Drawing Markup Tools — For circling parts of figures or spaces that require changes

These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Click on one of shape icons in the Commenting Toolbar
2. Draw the selected shape with the cursor
3. Once finished, move the cursor over the shape until an arrowhead appears and double click
4. Type the details of the required change in the red box



6. Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.



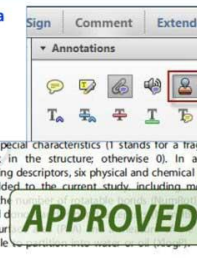
How to use it:

1. Right click on the Commenting Toolbar
2. Select "Attach a File as a Comment"
3. Click on paperclip icon that appears in the Commenting Toolbar
4. Click where you want to insert the attachment
5. Select the saved file from your PC or network
6. Select type of icon to appear (paperclip, graph, attachment or tag) and close

7. Approved Tool (Stamp) — For approving a proof if no corrections are required.

How to use it:

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2. Select the Approved rubber stamp from the „standard business“ selection
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Estimation of Group B *Streptococcus* Colonization in High-Risk Neonates by PCR and Standard Culture

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APPROVED

Background: Group B *Streptococcus* (GBS) (*Streptococcus agalactiae*) is the leading cause of morbidity and mortality of newborn infants considered as a leading factor causing septicemia after birth. The standard method for the diagnosis of GBS colonization is culture in a selective broth medium. Today, it is accepted that PCR has a high sensitivity and specificity in diagnosis.

Objectives: The goal of this study was to estimate the colonization of GBS in rectum of neonates of high-risk mother by culture and PCR method.

Patients and Methods: Samples were taken from rectal mucus of 154 neonates of high-risk mothers for GBS by swab. Samples were tested by standard culture using Todd Hewitt broth and blood agar and also by PCR using primers specific for *cfb* gene.

Results: Of 154 neonates, Culture identified 17 (11%) neonates as colonized by GBS; and the PCR assay could identify 27 (17%) neonates with positive results for GBS. Mothers age range was 17-40 years (mean = 26.1 ± 5.1). Maternal age was significantly lower in PCR positive group (P = 0.038) and in culture positive group (P = 0.015). Using culture as the gold standard, sensitivity, NPV, specificity, and PPV of PCR were 100%, 100%, 92%, and 62%, respectively. The time required for PCR assay and culture were 2 hours and 36 hours, respectively.

Conclusions: This study showed that incidence of GBS in Iranian high-risk neonates is high, so we strongly recommend screening of high-risk neonates for detection of GBS.

Keywords: *Streptococcus agalactiae*; Infant, Newborn; Colonization

1. Background

Group B *Streptococcus* is a facultative gram-positive diplococcus, which can colonize the vagina, rectum, urethra, umbilicus, ear and pharyngeal site. Risk factors for rectovaginal colonization are lower age, multi-sexual partners, and infrequent hand washing. Vertical transmission from colonized mothers to their infants occurs in 41-72% of cases, and only 1%-2% of colonized neonates develop invasive GBS disease, pneumonia and bacteremia in approximately 80% of cases, meningitis in 10%, and death in rare instances (4%) (1). GBS colonization in pregnant women may result in neonatal colonization and invasive disease; hence, maternal screening of GBS colonization seems to be crucial. Maternal factors associated with early streptococcal invasive disease in neonates are delivery before 37 weeks gestation, premature rupture of membrane more than 18 hours and intrapartum fever (2). There are different reports on GBS colonization in pregnant woman from Iran (22.7%) (3) (11.2%) (4) 10.2% (5) 3% (6)

and other regions such as 20% in Taiwan (7) 15.3% in Lithuania (8) and (6.2%) in China (9), this difference might be resulted from the number of partners and sexual habits in different cultures. Studies on neonatal colonization in Iran show different results. It was detected in 58.3% of dry umbilical cords (10) and 9.1% of tracheal secretions of intubated neonates due to respiratory distress in neonatal intensive care unit (NICU) (11).

This study offers a novel investigation to detect rectal colonization in neonates of high-risk pregnancies (before 37 weeks gestation, premature rupture of membrane more than 18 hours and intrapartum fever) (2) to determine the colonization of *Streptococcus* group B by culture and PCR method.

2. Objectives

The study hypothesis was to estimate the power of PCR in comparison to culture method in detection of GBS colonization in study cases.

Implication for health policy/practice/research/medical education:

Group B *Streptococcus* is an infectious pathogen, which colonizes vaginal and rectal region in women and may colonize their neonate. This pathogen may be the cause of sepsis in colonized neonates. This study offers a novel investigation for detection of rectal colonization in neonates of high-risk pregnancies (before 37 weeks gestation, premature rupture of membrane more than 18 hours and intrapartum fever) to determine the colonization of *Streptococcus* group B by culture and PCR method.

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3. Patients and Methods

This study was implemented on neonates of mothers with high-risk pregnancies from September 2009 to April 2010 in Imam Hossein Hospital (Tehran, IR Iran). Sampling was performed from neonatal rectal specimens during delivery by midwives educated on implementation of rectal swabbing. Informed consent was obtained from mothers. The study was approved by ethics committee of Shahid Beheshti University of Medical Sciences.

One hundred and fifty four neonates of high-risk mothers were included in the study. The exclusion criteria were GBS bacteriuria and planned cesarean section without membrane rupture in mothers. Sample size was calculated by considering a 15% positive result rate and delicacy of 0.05% resulted from 102 cases. Midwives obtained the specimens from neonate's rectum immediately after delivery. The double swab was placed into Stuart's transport medium (one for culture and the other for the PCR assay) and delivered to the microbiology laboratory (Pasteur Institute of Iran) in 12 hours.

3.1. Culture Identification of GBS

Swab specimens were plated on 5% sheep blood agar and then into the LIM selective enrichment broth (Todd-Hewitt broth with 15 µg/mL nalidixic acid and 10 µg/mL colistin). After overnight incubation, the broths were subcultured onto 5% sheep blood agar. Solid and liquid media were incubated at 35°C with 5% CO₂, and solid media were read daily for two days. GBS was identified as follows: the presence of catalase-negative, beta-hemolytic colonies and a positive reaction for the GBS antigen by a slide agglutination test (PathoDx; Diagnostic Products Corporation, Los Angeles, CA), as recommended by the CDC guidelines (12). Non hemolytic colonies were tested for the GBS antigen reaction, based on colony appearance and catalase-negative and bile esculin-sensitive reactions.

3.2. Nucleic Acid Extraction for PCR Assay

In total, 0.5 mL of selective broth was centrifuged and the pellet was extracted by PCR template purification kit according to the manufacturer protocol (Roche, Germany). Aliquots of the remaining incubated LIM broth were frozen at -70°C immediately after the antigen detection and PCR assays were performed with batched samples.

3.3. PCR Assay

For each reaction mixture, 5 µL of the extracted material was added to 20 µL of PCR reagent. Briefly, primers Strep B1 (5'-TTTCACCAGCTGTATTAGAAGTA-3') and StrepB2 (5'-GTTCCCTGAACATTATCTTTGAT-3') amplify a 153-bp fragment of the *cfb* gene (13). Each 20 µL reaction volume contained 2 µL 10x PCR buffer (Roche Diagnostics), 0.4 µM each primer, 0.1 U of uracil-DNA glycosylase (UNG; Roche Diagnostics), 2.0 mM MgCl₂, one unit of Taq DNA polymerase and 5 µL of DNA extract. As an internal con-

trol, all the extracted DNAs were checked by universal primers which amplify the conserved region of 23s rDNA (14). Purified DNA from a known GBS strain, confirmed by Lancefield Capillary Precipitin Methods (Gibco, USA) was used as positive control in two dilutions corresponding to 10⁴ and 10² bacterial genomes. As a negative control, sterile water was added instead of DNA template. A positive control and one or two negative controls were included in each run. Strict precautions to prevent carry-over of amplified DNA were used. Thermocycling conditions were optimized to one cycle at 95°C for 5 minutes, followed by 45 cycles of 45 seconds at 94°C, 45 seconds at 57°C and 45 seconds at 72°C. The amplified reaction products were analyzed by electrophoresis on 2% agarose gels.

3.4. Data Analysis

The information of maternal age, preterm < 37 weeks or term delivery, and culture and PCR results were recorded and analyzed by using SPSS (PASW Statistics 18), sensitivity, specificity, positive predictive value and negative predictive value and likelihood ratio calculation were performed and the confidence interval was calculated by Wilson Score Method. P value < 0.05 estimated by t-test was considered as significant.

Table 1. Culture and PCR Results in Different Groups of High-Risk Neonates^{a, b}

Groups	Culture +	PCR+	Culture PCR+
Preterm (n = 78)	8 (10)	12 (15)	12 (15)
PROM (n = 39)	5 (12)	8 (20)	8 (20)
Fever (n = 37)	4 (10)	7 (18)	7 (18)

^a Data are presented in No. (%).

^b Abbreviations: PCR, polymerase chain reaction; PROM, Premature Rupture Of Membrane

Table 2. PCR and Culture Results and Maternal Age in High-Risk Neonates^a

Test	Maternal Age	P value
PCR		0.04
+	24.3 ± 4.3	
-	26 ± 5.2	
Culture		0.015
+	23 ± 4.4	
-	26 ± 5.1	

^a Data are presented in Mean ± SD.

Table 3. Culture Results Based on Positive and Negative PCR Cases

	Culture+	Culture-
PCR+	17	10
PCR-	0	127

Table 4. Sensitivity, Specificity, PPV, NPV, LR+ and LR- Using Culture as the Gold Standard^a

Sensitivity	Specificity	PPV	NPV	Accuracy	LR+	LR-
100.00, %	92.70	62.96	100.00	93.51	13.7	0
81.57-100	87.08-95.99	44.23-78.47	97.06-100	88.46-96.43	7.543-24.882	0-0.385

^a Abbreviations: LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

4. Results

In total, 154 neonates entered the study based on the inclusion criteria, 39 cases were PROM (Premature Rupture of Membrane) (25%), 37 cases had fever (24%), and 78 cases had gestational age less than 37 weeks (51%), 27 cases (17%) were PCR positive and 17 cases (11%) were culture positive.

Twelve (15%) of preterm cases (78) and 8 (20%) of PROM cases and 7 (18%) of fever case (37) were PCR and culture positive, the number of culture positive cases were less than PCR positive cases in three groups, all culture positive cases were PCR positive simultaneously (Table 1). There was no significant difference between high-risk groups in PCR and culture positive and negative groups, Mothers age range was 17-40 years (mean = 26.1 ± 5.1). Maternal age was significantly lower in PCR positive group ($P = 0.038$) and in culture positive group ($P = 0.015$) (Table 2). Using culture as the gold standard, sensitivity, specificity, PPV, NPV, LR+ and LR- were 100%, 92.7%, 62.9%, 100%, 13.7 and 0 respectively (Table 3).

5. Discussion

The likelihood ratio of GBS infection in a neonate born to a mother with one or more risk factors being infected with GBS is about 3.9-fold higher than the background risk. The individual risk factors carry different 'weights', in which intrapartum fever is associated with the highest risk and prolonged rupture of membranes associated with the lowest risk (15, 16). This study estimated GBS colonization in rectum of Iranian neonates, and 17% PCR positive rate in neonates of high-risk pregnancy is high enough to be considered important and if other sites such as umbilicus and ear were added, the rate might be increased accordingly, so there may be probable missed cases of positive GBS colonization in our study. Higher colonization may result in higher sepsis rate in neonates, and increase in bacteriologic techniques sensitivity may result in higher positive cases of *Streptococcus* group B sepsis in our setting.

As a preventive strategy of neonatal sepsis with GBS, mothers colonized with GBS are recognized, and treated. Maternal GBS screening and intrapartum antibiotic prophylaxis showed that intrapartum antibiotic prophylaxis could reduce the incidence of early-onset GBS infection by 70 % (17). Different strategies are described to prevent GBS infection in neonates. Screening based strategy (antibiotic prophylaxis given to all colonized mothers) and

risk-based strategies (antibiotic prophylaxis given to all high-risk mothers) are implemented to reach this goal (18, 19). In resource-limited countries, implementation of culture, even in high-risk pregnant women is difficult, on the other hand, overuse of antibiotics in intrapartum period and neonates might be prevented. In a prospective study in Friuli-Venezia Giulia (north-eastern Italy), complete blood count and blood culture for GBS for all infants at risk, with a standardized physical examination had no advantage over only standardized physical examination on the onset of signs of possible sepsis and beginning the treatment, and significantly fewer infants were treated with antibiotics in the second group (20). Therefore, we need more investigations on GBS colonization and infection in our mothers and their neonates. Different methods for GBS screening are available, which are traditional culture and PCR, culture method is time consuming but more available, quantitative PCR may also offer the advantage of reduced time-to-results, and increased positive results, making it useful as an intrapartum screening method (21-23). Studies on different culture and PCR methods in maternal and neonatal detection of GBS colonization are in accordance with the results of our study. Among 375 women, with vaginal and rectal sampling, in Mirzakhochakhan Hospital Tehran, IR Iran (2011), 35 (9.3%) were identified as carriers of group B streptococci by cultures of specimens, compared to 42 (11.2 %) based on the PCR assay (4). Goodrich et al. compared routine culture and two real-time polymerase chain reaction (PCR) assays for detection of GBS: the light cycler (LC) Strep B analyte-specific reagents (ASRs) and the BD GeneOhm Strep B (BD-Strep B) test, detection rate increased from 3.0% to 3.5% with PCR, the sensitivity/specificity of the LC Strep B ASR was 100%/95.9%, and for BD-Strep B test was 92.5%/92.5% by using culture as the gold standard method (24). Gavino et al. performed both rapid test (PCR) and intrapartum culture on 55 subjects as GBS screening at 35-37 weeks of gestational age, GBS colonization rate was 43.6% by culture and 62% by PCR. Sensitivity and specificity of the PCR test were 95.8% and 64.5% respectively (23). Antenatal GBS screening was performed by Davis et al. using two combined vaginal/anal with rapid molecular diagnostic test (IDI-Strep B; infection diagnostic) and culture for intrapartum GBS detection after 36 weeks of gestational age in 5 North American centers during September 2001 to May 2002. In total, 881 women were recruited into the study. The overall intrapartum GBS colonization rate was 18.6%

in study sites. The sensitivity of IDI-Strep B in study sites ranged from 85% to 99%, and specificity ranged from 93% to 100% (22). Rallu et al. found culture positive rectovaginal specimens as screening tests from two hundred six LIM enrichment broth samples in 62 cases and PCR positive in 64 cases (25). In concern with the results of this study, we recommend to consider culture (more available) and PCR method to detect GBS colonization and infection in neonates of high-risk mothers. PCR method is preferable because of 100% PPV, but still we can rely on the result of culture because of its 100% specificity and very low false positive rate, although its lower sensitivity 62.9% shows a higher false negative rate. We recommend to consider group B *Streptococcus* as an important pathogen in neonatal sepsis and including GBS screening test in evaluation of high-risk pregnant women with higher chance of colonization with this organism to prevent the colonization and sepsis in their neonates.

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Authors' Contribution

[Will be written by author].

Financial Disclosure

The authors declare no competing financial interests and conflict of Interest.

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