Method for Quantitative Detection and Presumptive Identification of Group B Streptococci on Primary Plating

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Maternal prenatal screening for group B streptococci (GBS) followed by offering of intrapartum chemoprophylaxis to carriers is one of the strategies used to reduce the incidence of neonatal early-onset GBS infections. Culturing of vaginal and anorectal swab specimens in selective broth is the screening procedure recommended by the Centers for Disease Control and Prevention. This technique is sensitive; it does not, however, allow either evaluation of the degree of colonization or detection of cocolonization with several GBS clones. We have examined the carriage rate and population dynamics of GBS in a group of Danish women during pregnancy and 1 year after delivery using a new detection method. In the present paper we describe a mixed blood agar medium (MB agar) that identifies GBS in the primary cultures by detection of a double hemolysis pattern consisting of characteristic, large zones of partial hemolysis (“CAMP zones”) and of narrow zones of complete hemolysis. The MB agar was at least as sensitive as culturing in selective broth for detection of GBS in vaginal and anorectal swab specimens, and GBS strains could be identified directly on the primary plate due to the CAMP zones without the need for subculturing. The carriage rate of GBS in a group of Danish women was found to be more than 30%, a figure considerably higher than the rate that was reported previously.

Streptococcus agalactiae (group B streptococcus [GBS]) is the principal cause of neonatal infections in industrialized countries. A significant decline in the prevalence of neonatal GBS infections has been observed in the United States as a result of implementation of preventive measures (7, 28). These include increased use of chemoprophylaxis during labor in women at high risk for transmitting the infection to their newborns (29). Risk-based or screening-based strategies are recommended by the Centers for Disease Control and Prevention to prevent GBS disease (6). Collection of swab specimens at 35 to 37 weeks of gestation is suggested for the screening-based approach. Rectal and vaginal swab specimens are inoculated together in a selective broth for 18 to 24 h. The broth is subcultured on sheep blood agar for another 18 to 24 h, and suspected colonies are finally examined by conventional microbiological techniques. This procedure is not applicable to semi-quantitative evaluation of colonization intensity, and it is not feasible for demonstration of colonization with multiple clones.

For a longitudinal study of the rate of carriage and population dynamics of GBS in a cohort of pregnant women, we needed a medium that compensates for these shortcomings. We developed a solid medium based on a modification of the classical CAMP reaction that allows easy detection of individual colonies of GBS in primary cultures.

GBS generate a unique protein, the so-called CAMP factor, which interacts with the plasma membrane of red blood cells (RBCs) and other cell types. CAMP factor causes hemolysis of sheep RBCs (SRBCs) that have been altered by Staphylococcus aureus β-toxin ( sphingomyelinase). This phenomenon is designated the CAMP reaction, which refers to the authors Christie, Atkins, and Munch-Petersen (8). CAMP reaction-like reactions have also been observed after the combined actions of some other bacterial products on blood, and cohemolysis is therefore a more adequate expression for a hemolytic reaction induced by the synergistic actions of two different compounds, as discussed below. The CAMP factor of GBS has been characterized and has been renamed protein B. It has been suggested that the target of protein B is ceramide (N-acyl-sphingosine) (4), i.e., a membrane lipid generated from sphingomyelin by sphingomyelinase cleavage. Binding of protein B causes disorganization of the lipid bilayer of the cell membrane to an extent that results in cell lysis (4). The in vivo function of protein B has not been disclosed yet; however, the hemolytic activity seems to be an epiphenomenon (19, 27). Binding of this protein to the Fc region of immunoglobulins has been demonstrated (12, 19), and the protein seems to be lethal to rabbits and mice (30). Protein B seems to be essential for S. agalactiae, as the cfb gene encoding this protein (24) was found to be universally present in the genomes of 162 GBS strains from different geographic areas (20). Furthermore, 96 to 99% of all human GBS isolates have been found to be positive by the CAMP reaction (11, 14, 23, 25). Nearly all group A streptococci (Streptococcus pyogenes) and Streptococcus uberis strains possess equivalent genes named cfa and cfe, respectively (15, 18). The gene may also be present in some other streptococcal species (16, 18). The cohemolysis observed with other species is due to substances different from protein B. The CAMP reaction-like reaction of Listeria monocytogenes is caused by listeriolysin O (26), and that of Actinobacillus pleuropneumoniae is caused by an RTX toxin (17).

Protein B may cause lysis of β-toxin-modified RBCs, as mentioned above; however, only cells containing more than 45 mol% of sphingomyelin in the plasma membrane are sensitive to the combined actions of the staphylococcal toxin and pro-
tein B (31). Thus, the CAMP reaction is seen on agar plates prepared from bovine RBCs and SRBCs, whereas RBCs from humans, rabbits, and guinea pigs and horse RBCs (HRBCs) are not lysed (8, 31).

We have taken advantage of the unique feature of protein B as the indicative principle in a new blood agar medium that can be used for the easy, sensitive, and specific detection and enumeration of GBS in primary cultures of complex specimens such as anorectal and vaginal swab specimens. The agar plates described in this article contained a mixture of SRBCs and HRBCs, which were made sensitive to the action of protein B by pretreatment with sphingomyelinase. The method was successfully applied in a longitudinal study of the GBS carrier status of a group of Danish women during pregnancy and after delivery (unpublished data). Compared with five other techniques for GBS detection, the new medium was found to be at least as sensitive and easier to use, since subculturing was usually unnecessary. The option for semi quantitative enumeration of GBS and detection of coccolonization with several clones of GBS is an additional advantage of the mixed blood agar (MB agar) plates.

MATERIALS AND METHODS

Bacterial strains from collections. Two strains of GBS from our own collection were used for control purposes. Strain RH595 is beta-hemolytic and yields a positive CAMP reaction. Strain RH511A also yields a positive CAMP reaction but is nonhemolytic. Staphylococcal beta-toxin was prepared from cultures of an S. aureus strain (SSI AB2775) from the Department of Clinical Microbiology, Statens Serum Institut, Copenhagen, Denmark. The toxin was used for treatment of MB agar plates (see below).

Preparation of crude staphylococcal beta-toxin. Partly purified beta-toxin (sphingomyelinase) was prepared from a Todd-Hewitt broth culture (no. CM189; Oxoid Ltd., Basingstoke, United Kingdom) of the S. aureus strain as follows. The culture was incubated for 24 h at 37°C (optimum, 24 to 28 h), and the bacteria were removed by centrifugation (1,750 × g, 30 min). The clear supernatant was filter sterilized (pore size, 0.45 μm), and 104 g of (NH₄)₂SO₄ per liter was added. After the mixture was stirred with a magnetic stirrer for 1 h and allowed to settle for 30 min at room temperature, the solution was centrifuged (10,000 × g) for 20 min. The pellet of the precipitate was discharged, and another 104 g of (NH₄)₂SO₄ per liter was added to the total volume of the supernatant. After stirring of the mixture for 1 h, the mixture was kept at room temperature. On the next day, the solution was centrifuged (10,000 × g) for 20 min. The second supernatant was discharged, and the pellet of crude toxin was dissolved in 20 ml of sterile phosphate-buffered saline (PBS). The toxin concentrate was dialyzed four times against 2 liters of PBS for 24 h at 5°C. Finally, the concentrated toxin stock solution was filter sterilized (pore size, 0.45 μm) and stored at 5°C until it was used. The toxin preparation was stable for several months when stored at 5°C. The sphingomyelinase activity of the toxin stock was determined by titration. The whole surfaces of a number of MB agar plates (see below) were treated with 200 μl of twofold serial dilutions of the toxin stock in PBS. A CAMP reaction-positive strain of GBS (strain RH595) was streaked onto each of the toxin-treated plates before they were incubated overnight at 37°C. The plates were inspected for detection of two different hemolysis phenomena around colonies of GBS (double hemolysis). The action of beta-hemolysin caused narrow zones of complete hemolysis, and protein B caused larger zones of partial hemolysis (Fig. 1). The highest toxin dilution that gave optimal double hemolysis around the colonies of both bacterial strains; the SRBCs are lysed in these areas due to the interaction with protein B generated by the bacteria, whereas the HRBCs are left intact, as these cells are insensitive to protein B; 3, after removal of the bacterial colony, a narrow spot of complete hemolysis, in which both kinds of RBCs are lysed due to the action of beta-hemolysin, is seen in the middle of the CAMP zone; 4, as for arrow 3, except that the bacterial colonies were left in place; beta-hemolysis is seen as thin clear rings around the bacterial colonies; 5 and 6, no beta-hemolysis appears around the colonies of strain RH511A or after removal of a colony.

MB agar plates. Modified CAMP reaction plates containing mixed blood were prepared with a double layer of agar. One liter of agar contained 40 g of blood agar base (no. CM554; Oxoid), 2 g of sodium pyruvate, 0.247 g of MgSO₄, and 50 mg of l-cysteine hydrochloride monohydrate. The ingredients were mixed during heating to the boiling point and then autoclaved for 15 min at 121°C. The medium was cooled and kept in a water bath at 50°C. For preparation of the top layer, 2.5% (vol/vol) packed SRBCs and 2.5% (vol/vol) packed HRBCs were added to the 50°C agar base and mixed. The blood cells were washed three times before use in PBS (0.1 M NaCl, 0.05 M phosphate buffer [pH 7.4]) and packed by centrifugation (at 500 × g for 5 min). Each plate (diameter, 9 cm) was made of a lower layer of agar base without blood cells (25 ml). After solidification, a top layer of agar base (20 ml) containing 5% mixed blood cells was added. Untreated plates were stable and could be stored for several weeks in a refrigerator. Before use, the MB agar plates were pretreated with staphylococcal beta-toxin, as described below. After treatment with the staphylococcal toxin, the plates were somewhat sensitive to lowering of the temperature. To avoid spontaneous hemolysis, these plates were kept at room temperature and used on the same day.

g-MB agar plates. MB agar plates containing gentamicin (g-MB agar plates) in both the lower and the top layers were prepared by adding 1 ml of a filter-sterilized (pore size, 0.45 μm) stock solution (4 mg per ml of H₂O) of gentamicin sulfate (658 μg of gentamicin base per mg; no. G 6896; Sigma-Aldrich, St. Louis, Mo.) per liter of agar base at 50°C (see above).

ng-MB agar plates. MB agar plates containing both nalidixic acid and gentamicin (ng-MB agar plates) in both the lower and the top layers were prepared by adding 1 ml of a filter-sterilized (pore size, 0.45 μm) stock solution (8 mg per ml of H₂O) of gentamicin sulfate (no. G 6896; Sigma-Aldrich) and 1 ml of a
TABLE 1. Frequency of GBS in swab samples cultured on different media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Anorectal swab specimens (n = 312)</th>
<th>Vaginal swab specimens (n = 311)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
<td>Sensitivity (%)</td>
</tr>
<tr>
<td>Blood agar</td>
<td>27</td>
<td>25.2</td>
</tr>
<tr>
<td>MB agar</td>
<td>57</td>
<td>34.6</td>
</tr>
<tr>
<td>g-MB agar</td>
<td>88</td>
<td>82.2</td>
</tr>
<tr>
<td>ng-MB agar</td>
<td>101d</td>
<td>94.4</td>
</tr>
<tr>
<td>Selective broth</td>
<td>86</td>
<td>80.4</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td></td>
</tr>
</tbody>
</table>

* Total number of swabs tested on all media.
* Blood agar, 5% blood agar plates; MB agar, agar plates containing SRBCs and HRBCs and pretreated with staphylococcal β-toxin; g-MB agar, MB agar with gentamicin sulfate; ng-MB agar, MB agar with nalidixic acid and gentamicin sulfate; selective broth, Todd-Hewitt broth with nalidixic acid and gentamicin sulfate; total, number of swabs yielding growth of GBS on one or more of the media as used.
* Sensitivity, percentage of swabs that tested positive on the medium of the total number of swabs that tested positive on one or more of the media used.
* The number of anorectal swab specimens positive on ng-MB agar was significantly higher (P < 0.001) than the number of swabs positive in selective broth (see text).
* The number of vaginal swab specimens positive on ng-MB agar was not significantly different (P = 0.30) from the number of specimens positive in selective broth (see test).

Evaluation of intensity of GBS colonization on ng-MB agar plates. Presumptive identification of GBS colonies is possible directly on the ng-MB agar plates (see above). These plates were therefore used for semiquantitative evaluation of the degree of colonization, as follows. Upon arrival in the laboratory in Stuart’s transport medium, each swab specimen (vaginal or anorectal) was rolled and rubbed over an area 2 by 3 cm near the edge of an ng-MB agar plate. The inoculum was streaked from this area five to six times with one side of a sterile 10-μl plastic inoculation loop. To obtain well-separated colonies, spreading was continued by making three sets of streaks perpendicular to the previous streak by using the other side of the same loop. One person performed the inoculation of the agar plates to make it reproducible as possible. Colonization intensity was evaluated semiquantitatively and blindly for each plate after incubation for 18 h at 37°C in an atmosphere containing 5% CO₂. Growth of GBS in streaking area one or two only was considered light colonization, while growth of GBS in one or more of the following streaking areas was considered moderate to heavy colonization. Samples without detectable growth of GBS were considered negative.

Reagents. All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich.

Statistics. SigmaStat statistical software (version 1.0; Jandel Corporation, San Rafael, Calif.) was used for analysis of contingency tables by McNemar’s test.

RESULTS

Initial experiments revealed large zones of hemolysis around colonies of GBS grown on sheep blood agar plates pretreated with staphylococcal β-toxin. In contrast, only narrow zones of hemolysis were seen on plates not pretreated with β-toxin. The hemolysis around colonies of GBS grown on horse blood agar plates was unaffected by β-toxin pretreatment. On this medium the bacteria generated only narrow zones of hemolysis. A double pattern of partial and complete hemolysis was seen when the bacteria were cultured on β-toxin-treated agar plates with mixed SRBCs and HRBCs (MB agar; Fig. 1). The composition of the MB agar plates was optimized in a number of experiments to promote the most clear-cut reactions for GBS strains (the recipe is given in the Materials and Methods section).

Regular blood agar plates. Horse blood (5%) agar plates were purchased from the Statens Serum Institut. Selective broth. Todd-Hewitt broth (no. 189; Oxoid) was supplemented with 8.0 mg of gentamicin sulfate and 15 mg of nalidixic acid per liter (6). Cotton-stoppered test tubes containing 5 ml of this medium were autoclaved for 15 min at 121°C. After the swabs were streaked on the different solid media they were placed in this selective broth, mixed carefully on a vortex mixer, and incubated overnight at 37°C. A total of 10 μl of broth from each of the test tubes was streaked on regular 5% blood agar plates, and the plates were incubated overnight at 37°C.

Bacterial identification. Suspected isolates of GBS were identified by a combination of standard tests (21). The strains were Gram stained, tested for catalase activity, and examined by the traditional CAMP test on CAMP test plates (Statens Serum Institut). The strains were examined for the presence of the group B antigen by latex agglutination (no. ZL52; Streptex; Murex Biotech Ltd., Dartford, United Kingdom).

Clinical samples. A cohort of 77 healthy women was monitored in a longitudinal study (unpublished data) from the 19th week of gestation until shortly before delivery at the Department of Obstetrics, Aarhus University Hospital, Skejby, Denmark. All pregnancies were normal, and no complications due to GBS were observed. The project was initiated in January 1999 with approval from the Ethics Committee, County of Aarhus, Denmark, and after informed consent had been provided by all volunteers. The participants were instructed in the technique for obtaining swab specimens at home. Cotton-containing cotton swabs on plastic sticks (Statens Serum Institut) were used for the sampling. Vaginal and anorectal swab specimens were obtained at different times during the pregnancy and again 1 year after delivery (spring 2001). Each swab was immediately placed in a tube containing Stuart’s transport medium (Statens Serum Institut) and shipped to the laboratory by regular mail. All swabs were examined within 36 h after the samples were taken. For the present study, more than 300 paired samples were examined on up to five different media (Table 1). The swabs were inoculated on the solid media as described below for the ng-MB agar plates. Some samples were not examined on all media (exact figures are given elsewhere in the text).
TABLE 2. Frequency of GBS in 358 pairs of anorectal and vaginal swab specimens cultured on ng-MB agar

<table>
<thead>
<tr>
<th>Result for anorectal swab specimens</th>
<th>No. (%) of vaginal swab specimens</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
<td>107 (30)</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (4)</td>
</tr>
</tbody>
</table>

The number of anorectal swab specimens positive for GBS was significantly higher when ng-MB agar was used for cultivation than with cultivation in selective broth (355 swab specimens were tested on these two media; of these, 96 were positive on both media, 245 were negative on both media, 10 were positive on ng-MB agar and negative in selective broth, and 5 were negative on ng-MB agar and positive in selective broth [P = 0.30]).

As mentioned above, a strong correlation was found between the degree of colonization detected in the two swab samples (vaginal and anorectal) in a sample pair. For more than 90% of GBS-negative vaginal swab samples, the corresponding anorectal swab sample was negative as well (Fig. 2; Table 2). In contrast, among the GBS-positive vaginal swab samples, only 4 to 5% of the corresponding anorectal swab samples were negative. A clear tendency toward the detection of heavier colonization of the vaginal swab samples with increasing colonization of the corresponding anorectal swab samples was also seen (Fig. 2). Vaginal colonization with GBS therefore reflects the anorectal colonization density to a high degree.

DISCUSSION

The CAMP test is a standard procedure for the presumptive identification of S. agalactiae (GBS) (9, 11). In the traditional assay, a β-toxin-producing S. aureus strain is streaked across a blood agar plate made with SRBCs. A number of suspected strains of GBS are then streaked on the same plate perpendicular to the streak made with S. aureus. For most GBS strains an arrowhead-shaped hemolysis zone appears near the intersection with the S. aureus strain after incubation of the plate. This is due to the cohemolysis caused by the concerted action of β-toxin from the S. aureus strain and protein B from the strains of GBS. While this CAMP test is useful for the presumptive identification of pure cultures of suspected strains of GBS (9), the classical assay is not applicable for direct detection of GBS in primary cultures of clinical samples. Different modifications of the CAMP test have been developed for this purpose (3, 10). However, none of these methods are optimal for routine examination of a large number of samples, since the procedures are relatively complicated. Others have used sheep blood agar plates conditioned with sterile β-toxin-containing supernatant from an S. aureus culture for detection of CAMP test-positive GBS (1), but such agar plates cannot distinguish the CAMP reaction and ordinary β-hemolysis.

The Centers for Disease Control and Prevention recommended a procedure for screening for carriage of GBS that requires culture of anorectal and vaginal swab specimens in a selective broth which, after incubation, is subcultured on blood agar plates; the isolates are eventually identified by serological methods (6). This procedure is specific and sensitive but is somewhat laborious, costly, and time-consuming and does not allow quantitative evaluation and proportional isolation of the individual strains in one sample.

The selective MB agar has several advantages, although preparation of the MB agar plates is more complicated than preparation of the traditional plates. Culturing on MB agar was found to be at least as sensitive as culturing in a selective..
broth, with a sensitivity of over 90%. The primary solid media can be inspected directly within 18 h, and reculturing is rarely needed, since typical colonies can be identified as GBS with a high degree of confidence on the basis of the characteristic hemolysis phenomenon. The density of GBS colonization can therefore be estimated semiquantitatively. We found a clear correlation between the colonization densities of GBS in paired vaginal and anorectal samples by use of ng-MB agar plates (Fig. 2). Furthermore, individual bacterial colonies can be identified on the primary plates and may be selected for further examination (e.g., for antibiotic resistance testing, DNA analysis, or serotyping).

Some bacterial species other than GBS may exhibit a positive CAMP reaction, as mentioned above, but these species do not constitute a diagnostic problem when MB agar is used for cultivation of human clinical specimens. On this medium the hemolytic pattern of group A streptococci differs from that of GBS (see above). S. uberis is not associated with humans, and in contrast to most GBS strains, strains of this species are not beta-hemolytic (21). A. pleuropneumoniae is found only in pigs. Occasionally, stool samples may contain L. monocytogenes (2), and colonies of this species resemble colonies of beta-hemolytic streptococci on blood agar. However, L. monocytogenes is sensitive to gentamicin and will, therefore, not appear on the selective MB agar plates. Furthermore, differentiation from GBS is easily accomplished by demonstrating the positive catalase reaction of L. monocytogenes.

Among a group of 77 pregnant women, we found 21% to be persistent carriers of GBS and an additional 27% to be transient carriers of GBS. These rates are considerably higher than the rates previously reported for a large group of Danish pregnant women (13), in which only 8 to 15% carriers of GBS were found. Other recent rates of carriage are not available from Denmark. Our prevalence findings based on the use of selective MB agar are similar to those recently reported for younger university students, which were found to be colonized with GBS (5), and among a group of pregnant women in a prospective cohort study, 28% were found to be colonized with GBS (22).

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REFERENCES