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Comparison of Carrot Broth- and Selective Todd-Hewitt Broth-Enhanced PCR Protocols for Real-Time Detection of *Streptococcus agalactiae* in Prenatal Vaginal/Anorectal Specimens[▽]

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The reporting of accurate *Streptococcus agalactiae* screening results in a short time frame is of tremendous clinical benefit. A total of 203 consecutive primary vaginal/anorectal specimens were cultured in selective Todd-Hewitt broth (LIM broth) and with the StrepB carrot broth kit (carrot broth). One-day broth cultures were subjected to both centrifugation and clarification of a 500-μl aliquot prior to sample lysis (protocol A) and direct lysis of a 50-μl aliquot (protocol B). The lysates were subsequently analyzed by the BD GeneOhm StrepB assay. The results were compared to the carrot broth culture results derived from visualization of pigment on day 1 or from a subculture of carrot broth. Thirty-four carrot broth cultures (16.7%) generated diagnostic pigment following overnight incubation; an additional 26 (12.8%) were positive for *S. agalactiae* upon subculture. Carrot broth-enhanced PCR by the use of either protocol A or protocol B trended toward a higher rate of positive results (33.0%) than the rate observed by either the LIM broth-enhanced PCR (30.5%) or full carrot broth culture analysis (29.6%). In the context of the result on day 1, both carrot broth- and LIM broth-enhanced PCRs generated more true-positive results ($P < 0.001$) than carrot broth culture visualization. The predictive values for both protocols of carrot broth- or LIM broth-enhanced PCR were ≥95.4%. Whereas protocol A resolved the results for 99.8% of the specimens in the evaluation upon initial testing, a 5.7% initial unresolved rate and a 1.5% final unresolved rate were determined by the use of protocol B. The use of carrot broth within a rapid and highly accurate molecular reflex testing algorithm can limit follow-up testing to cultures without evidence of pigmentation.

Streptococcus agalactiae (beta-hemolytic *Streptococcus* group B) is a significant cause of morbidity and mortality among newborns in the United States. Colonization occurs in 15 to 40% of pregnant women (1, 2, 6, 8, 15, 45), contributing to a neonatal infection rate of 0.73 per 1,000 live births (13). Since studies have shown that intrapartum prophylaxis of colonized mothers can significantly reduce the rate of vertical transmission of the organism (7, 23; for a review, see reference 37), current Centers for Disease Control and Prevention (CDC) recommendations suggest that all pregnant women be screened for vaginal and rectal *S. agalactiae* colonization at 35 to 37 weeks of gestation (10). Microbiology-based screening techniques have become embraced as the most effective means of preventing the onset of perinatal *S. agalactiae* disease. Such methods include the CDC-advocated inoculation of Todd-Hewitt broth supplemented with nalidixic acid and colistin (LIM broth). However, these selective broth culture methods can be labor-intensive and may require up to 72 h before procurement of a final result.

Prenatal practice modifications have reduced the incidence of early-onset neonatal group B streptococcal disease in the United States by approximately 80% over the past 30 years (13, 19). One eight-state surveillance program reported a 65% reduction in the prevalence of early-onset disease from 1993 to 1998 (34). Despite this reduction, cases of early-onset disease continue to occur and are particularly prevalent in children born to mothers whose *S. agalactiae* carrier status is negative or unknown or who have received no prenatal care (28, 42). The diagnostic modalities used to combat this scenario have included antigen-based testing (3, 40) and nucleic acid amplification (14, 24) of primary clinical specimens. Others have proposed inoculation of an adjunct solid medium (16, 18, 43) or follow-up testing of enrichment broth (4, 30, 43), including methods that incorporate PCR (20, 24, 30).

The StrepB carrot broth kit (carrot broth; Hardy Diagnostics, Santa Maria, CA) has Granada medium as its basis (31) and generates an orange pigmentation upon overnight cultivation of beta-hemolytic *S. agalactiae* in a sufficient quantity. In the absence of visual color production, the broth is subcultured to solid medium which is observed for 48 h for the growth of *S. agalactiae*. Recent data have suggested that carrot broth is more sensitive than LIM broth for the detection of *S. agalactiae* (21, 35). It was hypothesized, therefore, that the performance of real-time PCR analysis with a clinical specimen that has been enhanced via overnight incubation in carrot broth could improve *S. agalactiae* detection to the point that a high percentage of the final results would be available 1 day after specimen collection.

(The results of this work were previously presented, in part, at the 108th General Meeting of the American Society for Microbiology, Boston, MA, 1 to 5 June 2008 [3a].)

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MATERIALS AND METHODS

Culture for *Streptococcus agalactiae*. Consecutive primary clinical vaginal/anorectal specimens submitted in the dual-swab Copan BBL CultureSwab collection and transport system (BD Diagnostics, Sparks, MD) for routine prenatal screen-

TABLE 1. Resolution of discrepancies generated by two experimental carrot broth- and LIM broth-enhanced *Streptococcus agalactiae* real-time PCR protocols

Specimen no.	Initial PCR result				Means of resolution[a]	Final interpretation
	Carrot broth enhanced		LIM enhanced			
	Protocol A	Protocol B	Protocol A	Protocol B		
1	+	+	−	−	FBRO	False-negative LIM protocols A and B
2	+	+	−	−	FBRO	False-negative LIM protocols A and B
3	+	+	−	−	SUBCULT, FLYS	False-negative LIM protocols A and B
4	+	+	−	−	SUBCULT, FBRO	False-negative LIM protocols A and B
5	+	+	−	−	FLYS	False-negative LIM protocols A and B
			+		FBRO	False-positive LIM protocol A
6	−	−		+	FBRO	False-positive LIM protocol B
7	−	−	−	+	FBRO	False-positive LIM protocol B
8	−	−	−	+	FLYS,[b] FBRO[c]	False-positive LIM protocol B
9	+	−	+	+	FLYS, FBRO	False-negative CB protocol B
10	+	−	−	−	FLYS	False-positive CB protocol A
11	−	+	−	−	FLYS, FBRO	False-positive CB protocol B

[a] FLYS, repeat testing of a frozen lysate; FBRO, testing of a frozen broth aliquot; SUBCULT, positive carrot broth subculture.
[b] Repeat testing of the frozen lysate by LIM-enhanced PCR protocol B yielded a positive result.
[c] Testing of a frozen broth aliquot by LIM-enhanced PCR protocol B yielded a negative result.

ing for *S. agalactiae* were evaluated. Separate swabs were placed into carrot broth (prepared according to the manufacturer's specifications) and LIM broth (Remel, Incorporated, Lenexa, KS). Both media were incubated at 35°C in ambient air. Following one overnight incubation, the carrot broth was observed for the characteristic pigment. In the absence of color development, tryptic soy agar plates containing 5% defibrinated sheep blood (blood agar; Remel) were inoculated with carrot broth aliquots and were incubated for 48 h at 35°C in 4 to 6% CO_2. Propagated colonies suspicious of being *S. agalactiae* were initially assessed by Gram and catalase reactions, and the identities were confirmed by serotyping (PathoDx Strep grouping kit; Remel) (46).

Centrifugation and clarification broth-enhanced PCR (experimental protocol A). Following one overnight incubation, fresh 500-μl aliquots of cryptically encoded carrot or LIM broth were centrifuged (14,000 × *g*, 5 min) in glass bead lysis tubes that are constituents of the BD GeneOhm StrepB assay (BD Diagnostics, Ste-Foy, Quebec, Canada). The supernatants were discarded and replaced with 50 μl kit-provided Tris-EDTA buffer. The lysis tube contents were then vortexed for 5 min, pulse centrifuged for 5 to 10 s, and subsequently incubated for 2 min on a 95°C heating block. The lysates were cooled for ≥10 min at 2 to 8°C.

Direct broth-enhanced PCR (experimental protocol B). Fresh 50-μl aliquots of the aforementioned carrot and LIM broths were delivered directly into glass bead lysis tubes. The contents were then vortexed for 5 min, pulse centrifuged for 5 to 10 s, and subsequently incubated for 2 min on a 95°C heating block. The lysates were cooled for ≥10 min at 2 to 8°C. Additional 1.5-ml aliquots of the carrot and LIM broths were frozen at −70°C for potential discrepancy analysis.

BD GeneOhm StrepB assay. Aliquots (1.5 μl) of the processed lysates were delivered to kit-provided SmartCycler reaction tubes containing a lyophilized master mix that was reconstituted with 25 μl of kit-provided diluent [Tris-HCl buffer, $(NH_4)_2SO_4$, $MgCl_2$]. All reaction tubes were pulse centrifuged for 5 to 10 s, inverted, vortexed for 10 to 15 s, and loaded onto a SmartCycler fluorescence thermal cycler for 37 cycles of PCR targeting a 154-bp fragment of *cfb*. Real-time amplicon detection occurred by hybridization of a FAM (6-carboxyfluorescein)- and DABCYL {4-[(4-dimethyamino)phenyl]azo benzoic acid}-based molecular beacon. The validity of each PCR result generated, in the face of the possible presence of medium-based inhibitors, was determined by the use of an internal control (a 134-bp DNA fragment not found in *S. agalactiae* that is flanked by the sequences of both *S. agalactiae*-specific primers) contained within the master mix. Following the setup of the reaction tube, the lysates were frozen at −20°C. Those lysates demonstrating amplification inhibition (unresolved result) were thawed and retested within 24 h.

Data analysis. The consensus PCR results generated by LIM broth- and carrot broth-based experimental protocols A and B constituted the reference results. Discordant PCR results emanating from comparisons of media for broth enhancement or from comparisons of the processing protocols were resolved in some instances by repeat testing of the frozen lysates. Clinical carrot broth culture results were utilized in additional scenarios as a reference standard for discrepancy analysis. Further circumstances warranted the processing of frozen aliquots of carrot and LIM broth by the use of protocols A and B. In one case (0.5% of all clinical specimens tested), majority rule (i.e., three common PCR results of the four results generated) was used to derive the reference result. The classification of false-negative and false-positive results ensued, with concomitant sensitivity, specificity, and predictive value calculations being performed.

Statistical analysis. The significance test of proportions (41) was used to determine if differences in the rates of positive *S. agalactiae* screening results or unresolved results were significant. The alpha level was set at 0.05 before the investigations commenced, and all *P* values are two tailed.

RESULTS

Comparison of carrot broth- and LIM broth-enhanced real-time *S. agalactiae* PCRs. A total of 203 consecutive primary clinical anal/vaginal specimens were cultured overnight for *S. agalactiae* in carrot and LIM broths and were subsequently analyzed by real-time PCR. A complete concordance of the results was observed for 192 (94.6%) of the specimens. Sixty-one of the specimens with concordant results (30.0% of the overall study set) indicated a positive PCR result for *S. agalactiae*. Five of the 11 specimens with discordant results (Table 1, specimens 1 to 5) involved a positive carrot broth-enhanced PCR result and a negative LIM broth-enhanced PCR result. Carrot broth culture correlation and/or PCR analysis of either frozen lysate material or frozen aliquots of broth medium verified these discordant results. One specimen with a discordant result (specimen 6) involved negative carrot broth-enhanced PCR and positive LIM broth-enhanced PCR results. Analysis generated negative results for the frozen broth aliquot and a subsequent false-positive interpretation for the LIM broth-enhanced PCR.

Comparison of experimental protocols for carrot broth- and LIM broth-enhanced real-time *S. agalactiae* PCRs. Five of the 11 specimens with discordant PCR results (Table 1, specimens 7 to 11) involved protocol-specific differences according to the medium used for broth enhancement. Discordant results were resolved by analysis of frozen lysate material and/or frozen aliquots of broth media, resulting in a consensus final result for four of the five specimens. For specimen 8, repeat testing of the frozen lysate derived from LIM broth-enhanced PCR pro-

TABLE 2. Performance and work-flow characteristics of *Streptococcus agalactiae* real-time PCR utilizing experimental carrot broth- and LIM broth-based enhancement protocols

PCR and protocol	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Rate of unresolved results (%)	
					Initial	Final
Carrot broth-enhanced PCR						
Protocol A	100	99.3	98.5	100	0	0.0
Protocol B	98.5	99.3	98.5	99.3	10.3	3.0
LIM broth-enhanced PCR						
Protocol A	92.5	99.3	98.4	96.4	0.5	0.5
Protocol B	92.5	97.8	95.4	96.4	1	0.0

tocol B generated a positive result, while analysis of a frozen aliquot of LIM broth (by protocol B) yielded a negative result. Four of the five protocol-specific discordant results (80%) were ascribed to the performance of protocol B.

Clinical accuracy of carrot broth- and LIM broth-enhanced real-time *S. agalactiae* PCRs. After addition of the results for specimens 1 to 5 (which had false-negative LIM broth-enhanced PCR results) and specimen 9 (which had a false-negative carrot broth-enhanced PCR result by the use of protocol B) to the results for the 61 specimens with concordant positive *S. agalactiae* PCR results, the overall rate of *S. agalactiae* molecular detection in this study was 33.0%. All performance indices related to the *S. agalactiae* PCR were greater than 98% when both carrot broth-enhanced PCR protocols were used (Table 2). In contrast, the LIM broth-enhanced PCR performed with either protocol had a positivity rate of 30.5%, subsequently resulting in lower values for the sensitivity and negative predictive value indices.

Work-flow parameters of carrot broth- and LIM broth-enhanced real-time *S. agalactiae* PCRs. On average, utilization of a centrifugation and clarification step (protocol A) added 1.3 min per specimen to the time required to process broth culture material for the real-time *S. agalactiae* PCR. The performance of either LIM broth-enhanced PCR protocol resulted in three cumulative initial unresolved results (a rate of 0.7% when data from both LIM broth-enhanced PCR protocols were combined). In contrast, the performance of the carrot broth-enhanced PCR resulted in 21 unresolved specimens on initial testing when data from both carrot broth-enhanced PCR protocols were pooled (rate, 5.2%). All unresolved carrot broth-enhanced PCR results were attributed to protocol B, although the inhibition rate was reduced significantly following one lysate freeze-thaw cycle ($P < 0.003$) (Table 2).

Comparison of carrot broth culture to carrot broth-enhanced real-time *S. agalactiae* PCR. Sixty-seven carrot broth-enhanced specimens were positive for *S. agalactiae* by real-time PCR. Of these specimens, only 34 (50.7%) yielded a positive result by carrot broth culture visualization ($P < 0.001$ versus the results for the carrot broth-enhanced PCR) (Table 3). Subculture of the remaining carrot broth tubes onto solid nutritive medium propagated 26 additional isolates typed as group B beta-hemolytic streptococci, yielding a final carrot broth culture sensitivity rate of 89.6% and a negative predictive value of 95.1% (Table 3). The time to the final result for the carrot broth culture from a starting time point of initial culture visualization averaged 36.7 h per specimen, which included an average of 3.7 min of microbiologist hands-on time (data not illustrated). No carrot broth culture-positive and carrot broth-enhanced PCR-negative specimens were encountered.

DISCUSSION

Guidelines for the prevention of perinatal *S. agalactiae* disease have evolved from a paradigm based, in part, on maternal risk factors (9) to one based on culture (10), largely as a result of data demonstrating the superiority of the latter approach (33). Recent studies have shown that the use of LIM broth enhancement culture yields 18 to 35% more clinical *S. agalactiae* isolates than culture with primary solid medium (18, 25). Carrot broth, a modification of Granada medium, is 8 to 15% more sensitive than LIM broth for detection of the organism (21, 35). However, a culture-based approach for the detection of *S. agalactiae*, especially one that requires subculturing to solid medium, does possess a number of limitations beyond the increased time to detection. Not all *S. agalactiae* strains exhibit beta-hemolysis (22). In addition, recovery of the organism can be compromised by competing saprophytic flora (16), feminine hygiene products (26), the site of specimen collection (15, 29), and circumstances related to specimen transport (39).

The development of rapid microbiological methods would greatly facilitate the antenatal care of women in a variety of clinical scenarios, including two that involve failure to screen for *S. agalactiae* within the recommended gestation window. First, in the context of preterm labor, associations between gestational age and early- and late-onset group B streptococcal disease have been reported (5, 37). Early-onset group B streptococcal disease case fatality rates are also inversely proportional to gestational age (34). Second, another population that misses the recommended *S. agalactiae* screening opportunity consists of women presenting for delivery without a history of

TABLE 3. Performance characteristics of carrot broth culture for *Streptococcus agalactiae* following overnight incubation and at time of final result for 67 PCR true-positive specimens

Time of carrot broth culture observation	No. of positive results	Sensitivity (%)	Negative predictive value (%)
After overnight incubation	34	50.7	80.5
After final subculture	60	89.6	95.1

prenatal care. Whitney et al. (42) used a multivariate analysis to show that the incidence of early-onset group B streptococcal disease increased with the number of women who did not receive prenatal care ($P = 0.01$). African-American women have a higher probability of receiving inadequate prenatal care (17), with a large percentage being unsure of their *S. agalactiae* screening status (12). African-American women also have a higher rate of *S. agalactiae* colonization than women of other races and ethnicities (8, 32, 38), and studies have demonstrated a greater likelihood of early-onset group B streptococcal disease in African-American neonates (11, 42). Schuchat et al. (36) demonstrated that this disease association was independent of other risk factors.

Women presenting for full-term delivery constitute a third demographic group for rapid *S. agalactiae* screening. In a retrospective 7-year review of early-onset group B streptococcal disease following the initiation of a microbiology screening-based protocol, Puopolo et al. (28) determined that the vast majority of cases (68%) occurred in term infants. Among those infants, 82.4% were born to mothers whose prenatal *S. agalactiae* screening status was determined to be negative. One factor that potentially explains the inaccuracy of these screens is transient colonization with *S. agalactiae* during pregnancy (6, 15, 45). It has been demonstrated that 4 to 9% of women with a negative screen in the later stages of the third trimester of gestation are colonized at delivery (6, 45). A second contributory factor may have been the utilization of a less-sensitive culture methodology during late-third-trimester screening.

In response to the demand for a highly accurate method for screening for *S. agalactiae* with an improved turnaround time, several approaches have been attempted. The direct detection of *S. agalactiae* antigen from primary clinical specimens by techniques such as optical immunoassay, latex agglutination, and enzyme immunoassay has demonstrated suboptimal sensitivity (3, 25, 40). Addition of a solid medium to the CDC-recommended broth culture resulted in 50 to 85% of test results being positive within 24 h (16, 18, 43). In the context of a culture reference standard, the colorimetric aspect of carrot broth has variable success in the provision of a final positive result after a single overnight incubation, with rates ranging from 80.8% in our laboratory to 94.3% in a multicenter study (35). Finally, while it is not explicitly advocated by the recent CDC recommendations (10), the detection of *S. agalactiae* has been accomplished by follow-up testing of an incubated broth culture. Nucleic acid hybridization of LIM broth culture contents identified *S. agalactiae* as accurately as traditional subculture (4, 43). Rallu et al. (30) reported that antigen detection of LIM broth contents was 15% more sensitive for the detection of *S. agalactiae* than the completion of a LIM broth subculture. Furthermore, nucleic acid amplification of LIM broth contents demonstrated a 12 to 57% greater sensitivity than the completion of a LIM broth subculture for the detection of *S. agalactiae*, but this was dependent upon the gene target and the commercial platform used (20, 30).

Rapid *S. agalactiae* screening results could also be generated by direct nucleic acid amplification testing of primary genital swab specimens. For a representative subset (based on the *S. agalactiae* isolation rate) of specimens within our study for which the performance of the BD GeneOhm StrepB assay directly with clinical swabs was compared to that of carrot broth culture ($n = 46$), the sensitivity of the direct swab PCR was only 59.1% (data not illustrated). An initial report by Hutchens and Schreckenberger (21), which included only 14 specimens positive for *S. agalactiae*, reported that the IDI-StrepB Cepheid Strep B SmartCycler assay had a sensitivity of 85.7% when it was performed directly with swab specimens compared to the results of carrot broth culture. Taken together, these data appear to be contrary to those from past studies of *S. agalactiae* PCRs performed directly with swab specimens (14, 24). Those studies reported sensitivity levels that approached 94%, which may be explained by the less sensitive LIM broth reference standard employed.

Given the enhanced sensitivity of carrot broth culture and reports of the increased sensitivity of LIM broth-enhanced PCR, we utilized a substantial set of clinical samples to investigate the potential benefit(s) of the carrot broth-enhanced PCR. Indeed, the rate of *S. agalactiae* detection derived from the carrot broth-enhanced PCR (33.0%) trended higher than the rates generated by carrot broth culture (29.6%) or LIM broth-enhanced PCR (30.5%). While these differences were not significant ($P > 0.45$), they are somewhat contrary to data from a smaller-scale study (21) that revealed equal sensitivity indices (100%) for carrot broth culture and carrot broth-enhanced PCR.

The direct broth-enhanced PCR (protocol B) simply involved the removal of 50 μl of broth culture for immediate physically and thermally induced cell lysis. This protocol proved to be effective in terms of both the time required (3.8 min per specimen) and the recovery of nucleic acid. However, several data suggest that the centrifugation and clarification broth-enhanced PCR (protocol A) may be superior to protocol B. The analytical sensitivity of the carrot broth-enhanced PCR with protocol A was 100%, whereas it was 98.5% when protocol B was used (Table 2). Specimen 9 (Table 1) yielded a positive result with a 500-μl aliquot (protocol A) but not with a 50-μl aliquot (protocol B). These data suggest that differences in sample volumes, especially from clinical material with a lower organism burden, may ultimately affect the ability to detect *S. agalactiae*. This may be offset by the utilization of a processing protocol that is based on a larger sampling volume. A 10.3% initial unresolved specimen rate was observed with the direct broth-enhanced PCR protocol, but this was significantly reduced after one lysate freeze-thaw cycle ($P = 0.003$). Utilization of a centrifugation and clarification step resulted in no unresolved specimens and only an additional 1.3 min of processing time per specimen. A previous report of carrot broth-enhanced PCR (21) yielded a 9.8% false-positive *S. agalactiae* detection rate, although the processing protocols were not clearly defined. Our larger-scale study did not reveal noteworthy specificity issues.

Our data indicate not only that the carrot broth-enhanced PCR is more sensitive than direct swab PCR but also that the method detects more *S. agalactiae* organisms than the conventional carrot broth culture method. As such, this paradigm may effectively address the *S. agalactiae* colonization status of the three aforementioned at-risk demographic groups. Rallu et al. (30) reported an improved sensitivity of LIM broth-enhanced PCR over that of conventional LIM broth culture. Yet, that group expressed concern over the utilization of this molecular-based methodology due to the potential overuse of intrapar-

tum antimicrobial prophylaxis, citing a paucity of studies relating very low detectable levels of *S. agalactiae* colonization to neonatal disease. The consequences of maternal *S. agalactiae* colonization status extend beyond intrapartum chemoprophylaxis. Peralta-Carcelen et al. (27) demonstrated an increase in pediatric services provided to asymptomatic term newborns born to risk factor-negative, *S. agalactiae* screen-positive mothers and alluded to a potential consequential induction of antimicrobial resistance. However, the findings presented in the aforementioned report from Puopolo et al. (28) and the observations of Yancey et al. (44) implicating a low colonization burden in subsequent disease argue in favor of the importance of such highly sensitive protocols. Concerns expressed by Rallu et al. (30) also apply to carrot broth-enhanced PCR; however, the establishment of a correlation between this experimental method of PCR detection and neonatal disease was beyond the scope of the present investigation. Additional studies may be warranted to determine the overall cost-effectiveness of this approach and the subsequent effect of the use of this approach on antimicrobial resistance patterns.

In summary, in light of the high negative predictive value and increased sensitivity associated with the PCR modification described here, carrot broth-enhanced PCR improves the accuracy and turnaround time of an *S. agalactiae* screening result, particularly when a centrifugation and clarification protocol is utilized. When the broth-enhanced PCR is combined with the overnight colorimetric determination inherent to carrot broth culture, the broth-enhanced PCR presents a molecular reflex testing algorithm that may further assist with the clinical management of both full-term delivery and preterm delivery.

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