Heterogeneity of Hemolysin Expression during Neonatal *Streptococcus agalactiae* Sepsis

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The β-hemolysin of *Streptococcus agalactiae* is a major virulence factor; consequently, nonhemolytic strains rarely cause infections. We report on a case of neonatal sepsis caused by a strain displaying heterogeneous hemolysin expression. It was detected by the simultaneous isolation of hemolytic and nonhemolytic colonies from cultures of the infant’s blood.

CASE REPORT

A term neonate was born at 41 weeks of gestation weighing 4,300 g. Due to a failure of adequate labor progress and pathological cardiotocographic readings, the infant was delivered by vacuum extraction. During delivery green amniotic fluid was noted. The Apgar score was 7/9/9, and the umbilical arterial pH was 7.17. Due to progressing signs of hypoxia, a gray skin color, a prolonged capillary refill time, and a further fall in the pH to 7.02, the child was transferred to the neonatal intensive care unit of the Pediatrics University Clinics 2 h after birth. The prenatal group B streptococcus colonization status of the mother was unknown. At admission, a blood sample for aerobic culture (PLUS Pediatric; BD, Heidelberg, Germany) was obtained and treatment with mezlocillin (200 mg/kg of body weight/day) and gentamicin (5 mg/kg/day) was initiated for suspected neonatal sepsis. The infant was negative for C-reactive protein (CRP) at admission, but the interleukin-8 level was elevated to 1,517 ng/liter. The aerobic blood culture bottle registered positive after 24 h of incubation in an automated blood culture system (Bectec 9240; BD). Microscopic analysis revealed the presence of gram-positive cocci growing in chains and displaying a typical streptococcal morphology. Following subculture on 5% sheep blood agar plates and overnight incubation at 35°C, the growth of two different colony types was confirmed by CAMP testing and serological testing. Targeted mutagenesis of the translytic strain was considerably larger than the same PCR product generated from the hemolytic blood culture isolate. The sizes of the PCR products of all the other colony types were indistinguishable from the sizes of the PCR product of the nonhemolytic isolate, genes of the *cyl* gene cluster were amplified by PCR, as described previously (12). Interestingly, the PCR product of the *cyl* gene amplified from the nonhemolytic strain was considerably larger than the same PCR product generated from the hemolytic blood culture isolate. The sizes of the PCR products of all the other *cyl* genes from the hemolytic and the nonhemolytic isolate were indistinguishable by visual inspection of the agarose gels and matched the expected sizes of the corresponding genes (Fig. 1). The *cyl* PCR product of the nonhemolytic isolate was further investigated by DNA sequencing, which revealed the presence of the insertion element IS1381 after nucleotide 467 of the *cyl* gene. *CylA* represents the ATP binding component of the *S. agalactiae* hemolysin transporter. Targeted mutagenesis of the *cyl* gene cluster in *S. agalactiae* via a homologous recombination strategy revealed the presence of a nonhemolytic strain that was not hemolytic.

During the next days the infant’s clinical situation quickly improved. The CRP concentration reached a maximum of 19.7 mg/liter. The cerebrospinal fluid did not reveal any signs of meningitis, and the child was discharged on day 4 in excellent clinical condition.

To assess the colonization status of the mother, a vaginal swab was obtained postpartum and was cultured in selective LIM broth, as recommended by CDC guidelines (11). Typical beta-hemolytic colonies were detected, and species identification revealed *S. agalactiae*. Despite a thorough inspection of the subcultures for evidence of nonhemolytic *S. agalactiae* colonies, only the beta-hemolytic phenotype could be isolated from the maternal vaginal tract.

To investigate the genetic basis for the loss of hemolysis in the nonhemolytic strain, genes of the *cyl* gene cluster were amplified by PCR, as described previously (12). Interestingly, the PCR product of the *cyl* gene amplified from the nonhemolytic strain was considerably larger than the same PCR product generated from the hemolytic blood culture isolate. The sizes of the PCR products of all the other *cyl* genes from the hemolytic and the nonhemolytic isolate were indistinguishable by visual inspection of the agarose gels and matched the expected sizes of the corresponding genes (Fig. 1). The *cyl* PCR product of the nonhemolytic isolate was further investigate by DNA sequencing, which revealed the presence of the insertion element IS1381 after nucleotide 467 of the *cyl* gene. *CylA* represents the ATP binding component of the *S. agalactiae* hemolysin transporter. Targeted mutagenesis of the *cyl* gene cluster in *S. agalactiae* via a homologous recombination strategy revealed the presence of a nonhemolytic strain that was not hemolytic.

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the hemolytic strains by more than just one additional band. Therefore, it remains unclear if all of the changes can be attributed to the insertion of an additional IS\textsubscript{1381} copy or if additional mutations account for these differences. IS\textsubscript{1381} is an insertion element that was first detected in \textit{Streptococcus pneumoniae} (10) and that is commonly found in clinical \textit{S. pneumoniae} strains. Its host range is, however, not limited to \textit{S. pneumoniae}, and it has been described as a potential tool for the subtyping of \textit{S. agalactiae} isolates (13).

To elucidate if the nonhemolytic strain did originate from the hemolytic strain found in the blood culture of the infant and the maternal vaginal tract, we performed molecular subtyping of all three strains. The capsular serotype was detected as described by Kong et al. (8) and showed that all three isolates were serotype III strains. Surface protein antigens were detected by PCR (2) and showed the presence of \textit{rib} in all strains. Further subtyping was achieved by multilocus sequence typing (MLST) (7) and revealed that both beta-hemolytic strains belong to sequence type (ST) 19 (ST19), which represents a major serotype III subclone. Interestingly, the nonhemolytic isolate belonged to ST27, which differs from ST19 only by a single nucleotide exchange in the \textit{glnA} gene. ST27 is therefore regarded as a member of the ST19 complex, which consists of several closely related STs (4). The fact that, aside from the loss of hemolysis, a point mutation occurred in the nonhemolytic strain underlines the idea of a high mutation rate in the respective beta-hemolytic blood culture isolate. The \textit{glnA} gene itself has not been associated with a loss of hemolysis. The vast majority of \textit{S. agalactiae} strains carrying allele 4 of \textit{glnA}, which is found in ST27 strains, are hemolytic. In summary, all subtyping methods supported the conclusion that the nonhemolytic strain represents a spontaneous mutant of the beta-hemolytic blood culture isolate (Table 1).

Mutations of \textit{S. agalactiae} isolates caused by the insertion of IS\textsubscript{1381} have repeatedly been reported (3, 9), and the presence of different copy numbers of IS\textsubscript{1381} elements in pairs of maternal-neonatal strains is not unusual (13). It is interesting to speculate when the insertion of the additional IS\textsubscript{1381} element in the \textit{cylA} gene occurred in our case. Thorough investigation of the vaginal swab from the mother only revealed a strain displaying the typical beta-hemolytic phenotype of \textit{S. agalactiae}. No evidence for the presence of a nonhemolytic strain was found in the vaginal swab material. Molecular typing showed the maternal strain to be identical to the beta-hemolytic blood culture isolate of the infant. The most likely scenario is therefore the transmission of the maternal beta-hemolytic strain from the mother to the infant and the subsequent invasion of the beta-hemolytic strain into the vascular system. Mutation of the \textit{cylA} gene through the insertion of an additional IS\textsubscript{1381} copy probably occurred afterwards. This hypothesis would be consistent with the fact that \textit{\beta}-hemolysin is regarded as a vir-

![FIG. 1. PCRs of \textit{S. agalactiae} cyl genes, as described previously (12). PCR was performed with the genomic DNA of nonhemolytic blood culture isolate BSU25 and hemolytic blood culture isolate BSU26. Lane M, molecular size marker. Genes that were amplified by a specific PCR are indicated. The numbers on the left are in nucleotides.](image1)

![FIG. 2. Southern blot analysis for the presence of IS\textsubscript{1381} in both neonatal strains (strains BSU25 and BSU26) and the maternal vaginal isolate (strain BSU27). An IS\textsubscript{1381}-specific probe was amplified with primers 5'-CTT GTT GGT GTT CAG CGC AC-3' and 5'-TGA ACA ACT TCA TAT CGT GGC-3'. Southern blotting was carried out after digestion of the genomic DNA with EcoRI and BamHI. Lane M, molecular size marker; lane 1, strain BSU25 (nonhemolytic neonatal isolate); lane 2, strain BSU26 (hemolytic neonatal isolate); lane 3, strain BSU27 (hemolytic maternal isolate). The numbers on the left are in nucleotides.](image2)

### TABLE 1. Molecular characteristics of the \textit{S. agalactiae} isolates\textsuperscript{*}

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Hemolysis</th>
<th>No. of IS\textsubscript{1381}-specific bands\textsuperscript{a}</th>
<th>Surface protein</th>
<th>Serotype</th>
<th>MLST type\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSU25</td>
<td>Neonatal blood culture</td>
<td>Nonhemolytic</td>
<td>6</td>
<td>Rib</td>
<td>III</td>
<td>27</td>
</tr>
<tr>
<td>BSU26</td>
<td>Neonatal blood culture</td>
<td>Beta-hemolysis</td>
<td>5</td>
<td>Rib</td>
<td>III</td>
<td>19</td>
</tr>
<tr>
<td>BSU27</td>
<td>Maternal vaginal tract</td>
<td>Beta-hemolysis</td>
<td>5</td>
<td>Rib</td>
<td>III</td>
<td>19</td>
</tr>
</tbody>
</table>

\textsuperscript{*}All strains were capsular serotype III, as detected by PCR (8). The surface protein of all isolates was Rib, as determined by multiplex PCR (2).

\textsuperscript{a}IS\textsubscript{1381}-specific bands were detected by Southern blot hybridization.

\textsuperscript{c}MLST sequence type determination was performed as described previously (7).
ulence factor important for the invasion of host tissues (5). It seems unlikely that the hemolytic strain and the nonhemolytic strain independently invaded the vascular system, especially since no evidence for the presence of a nonhemolytic strain in the maternal vaginal tract was found. However, we cannot, of course, exclude this possibility.

The identification of nonhemolytic \textit{S. agalactiae} isolates in blood cultures is rare, since these strains are of reduced virulence and the hemolysin is considered important for invasion processes. Among colonizing strains, less than 5\% of strains are nonhemolytic (1). The identification of nonhemolytic \textit{S. agalactiae} isolates is complicated by the unusual phenotype but can be achieved on the basis of biochemical tests and the CAMP reaction, which is positive for nonhemolytic strains. This is, to the best of our knowledge, the first report on the simultaneous detection of a hemolytic and a nonhemolytic \textit{S. agalactiae} strain in invasive infections.

REFERENCES