Distinctive Features of Surface-Anchored Proteins of *Streptococcus agalactiae* Strains from Zimbabwe Revealed by PCR and Dot Blotting

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Received 28 March 2008/Returned for modification 29 May 2008/Accepted 18 July 2008

The distribution of capsular polysaccharide (CPS) types and subtypes (serovariants) among 121 group B streptococcus (GBS) strains from Zimbabwe was examined. PCR was used for the detection of both CPS types and the surface-anchored and strain-variable proteins Cα, Cβ, Alp1, Alp2, Alp3, R4/Rib, and Alp4. The R3 protein was detected by an antibody-based method using monoclonal anti-R3 antibody in dot blotting. The CPS types detected, Ia (15.7% of strains), Ib (11.6%), II (8.3%), III (38.8%), V (24.0%), and nontypeable (1.7%), were essentially as expected on the basis of data from Western countries. The type V strains showed distinctive features with respect to protein markers in that Alp3 was detected in only 6.9% of the isolates while R3 occurred in 75.9% and R4/Rib occurred in 37.9% of the isolates. R3 occurred nearly always in combination with one of the alpha-like (Alp) proteins, and it was the third most common of the proteins studied. These results show that type V GBS strains from Zimbabwe differed from type V GBS isolates from other geographical areas and also emphasize the importance of the R3 protein in GBS serotyping and its potential importance in the immunobiology of GBS, including a potential role in a future GBS vaccine.

Group B streptococci (GBS) are a major cause of neonatal disease and may also affect adults, notably immunocompromised individuals. In many Western countries, much information on incidence and other epidemiological data on infectious diseases caused by GBS are available. Less is known with respect to incidence and other aspects of GBS disease in African countries, but experienced clinicians consider GBS infections an important cause of neonatal disease, for instance, in Zimbabwe (K. J. Nathoo, personal communication). In South Africa, the burden of neonatal GBS disease during the period from 1997 to 1999 was 2.06 cases/1,000 live births and 1 case/1,000 live births for early- and late-onset disease, respectively (19). In epidemiological settings, serotyping has been extensively used to identify and trace GBS variants and, in more recent years, has been supplemented or replaced with gene-based methods, such as multilocus sequence typing, pulsed-field gel electrophoresis, restriction endonuclease digestion pattern determination, and multilocus enzyme electrophoresis, albeit some investigators have considered these methods inadequate for determining GBS relatedness as described by whole-genome analysis (29).

The serotyping of GBS is based primarily on nine different capsular polysaccharide (CPS) antigens, called Ia, Ib, and II through VIII. Recently, a new CPS type, serotype IX, has been detected (25). Strains within each CPS type may also be subdivided into subtypes or serovariants on the basis of the expression of strain-variable and surface-anchored protein antigens or the detection of the genes encoding these proteins. These antigens include members of the alpha-like (Alp) protein family, Cα (bca), Alp1 (alp1), which is closely related to Cα and was previously called epsilon (GenBank accession no. U33554), Alp2 (alp2), Alp3 (alp3), and Rib (rib) (18), the latter being considered identical to the classical R4 protein (26). Alp proteins possess chimeric sequences, show variable immunological cross-reactivities, have repetitive structures, show ladder-like banding patterns on Western blots, and induce antibodies which are protective in experimental models (2, 16, 18, 28). The recently defined Alp4 (14), R5 (group B streptococcal protective surface protein) (9), and R3 (31) proteins have not been characterized to the same extent as the proteins mentioned above. In addition, the nonladdering CB protein, known for its ability to bind to immunoglobulin A (IgA) Fc fragments, also shows strain-variable expression (18).

In an earlier study, the serotype and serovariant distribution of GBS from Zimbabwe was determined by using antibody-based methods (23). The study disclosed that the CPS type distribution is essentially similar to that recorded previously for GBS from many Western countries, with some exceptions. A very low prevalence of strains which expressed the R1 protein, which probably includes the recently named Alp2 and Alp3 (16, 21), and a high prevalence of isolates which expressed the R3 protein were noticeable (23). The isolates studied had been collected in the early 1990s. In the present study, more recently collected Zimbabwean GBS isolates were serotyped, mostly by using molecular methods, to confirm or invalidate the conclusion that GBS from Zimbabwe have serotype marker characteristics somewhat different from those of GBS from many other areas. Such mapping is important...
both in epidemiological contexts and in the context of possible future GBS vaccine formulations.

MATERIALS AND METHODS

Bacterial strains. The reference and prototype strains used in this study have been described previously (15, 22). The isolates included strains of all known CPS types except serotype IX and isolates which expressed one or more of the GBS proteins Cβ, Co, Alp1, Alp2, Alp3, Alp4, R4/Rib, and R3. All of the strains belonged to the strain collection of the Laboratory of Medical Microbiology, St. Olavs Hospital, Trondheim, Norway. The 121 clinical Zimbabwean GBS strains under investigation were vaginal isolates from pregnant women (n = 109) and isolates from colonized neonates (n = 12), 6 of the latter from the ear and 6 from the umbilicus. All individuals were carriers without signs of GBS disease. The sampling and culturing were undertaken during the period from 2003 to 2005. Swabs were transported in Stuart’s transport medium and were cultured on tryptose blood agar base with 10 mg of colistin sulfate/liter and 15 mg of nalidixic acid/liter at 37°C for 18 h, after which serogroup determination was performed by means of the Pasteur grouping kit according to the instructions of the kit manufacturer (Bio-Rad, Marne-la-Coquette, France). Isolates were preserved in Greave’s medium at −70°C. The isolates were collected at the Chitunga (n = 25), Harare (n = 47), and Guruve (n = 49) health centers in Zimbabwe, meaning that they came from mainly rural, urban, and rural-urban areas, respectively.

Oligonucleotide primers. All oligonucleotide primer pairs for the identification of CPS-synthesizing genes (cps) were constructed by Eurogentec, S.A. (Liege, Belgium), as described by other investigators (4, 13). Primer sets constructed as described by Kong et al. (13) were as follows: for CPS Ia detection, the pair CpsA-LacpsHS1 (amplicon size, 354 bp); for CPS type Ib detection, the set BCps1SL-BcpsPs1A (523 bp); for CPS type III detection, ICpsHS-cpsIA (641 bp); for CPS type IV detection, ICPSHS1-ICPSMA (379 bp); for CPS type V detection, VcpsHS2-VcpsMA (374 bp); and for CPS type VI detection, VcpsHS1-VcpsLSA (360 bp). For PCRs to detect the CPS types II, VII, and VIII, primer pairs as described by Borchardt et al. (4) were constructed. The sizes of the amplicons generated by these PCRs were 590, 570, and 470 bp, respectively. Primers for use in multiplex PCR for the detection of protein antigen genes were constructed as described by Creiti et al. (5). The target genes included bca, rbi, alp1, alp2, alp3, and alp4, encoding the proteins Cβ, R4/Rib, Alp1, Alp2, Alp3, and Alp4, respectively. In these PCRs, a single forward primer targeted a conserved site in the genes and the reverse primers targeted gene-specific sites (5). For this reason, GBS markers defined by serology and those defined by the identification of genes encoding the markers are not distinguishable in this work.

The gene encoding the R3 protein has not been sequenced. Consequently, R3 expression was detected mostly by using the anti-R3 MAb in dot blotting. The MAb showed positive results in dot blotting for the R3 reference strain ATCC 49447 (10/84; V/R3) and strain ATCC 9828 (Compton, or Prague 25/60; nontypeable [NT]/R3, Alp4) and negative results for all of our other prototype and reference strains. In whole-cell-based Western blotting, the binding of the R3 MAb was recorded only when probing was done against the R3-expressing isolate (Fig. 1, lane 4). This was also the case for the polyclonal anti-R3 antibody which had been rendered R3-specific by cross-absorption (data not shown). These results confirmed the R3 specificity of both of the antibodies described previously (15).

RESULTS AND DISCUSSION

Performance of the tests. The performance of the CPS and protein gene PCRs was evaluated by testing isolates of our collection of reference and prototype GBS strains. Isolates of all GBS CPS types except the CPS type III (25) and isolates expressing one or more of the strain-variable proteins searched for in this study were tested. These strains showed PCR results as expected on the basis of known genotypic and phenotypic traits of the isolates. Thus, the testing confirmed the overall agreement between the results obtained by antibody-based serotyping of GBS and those obtained by molecular serotyping methods described by other investigators (4, 5, 6, 13, 14). For this reason, GBS markers defined by serology and those defined by the identification of genes encoding the markers are not distinguishable in this work.

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CPS type distribution. The distribution of CPS types among 121 Zimbabwean carrier GBS isolates is shown in Table 1. A total of 119 (98.3%) of the strains turned out to belong to one of five CPS types, Ia (15.7%), Ib (11.5%), II (8.3%), III (38.8%), and V (24.0%). Only two (1.6%) of the isolates were CPS NT. Overall, the CPS type distribution was similar to that recorded in previous testing of GBS from Zimbabwe (23) and also matches that among GBS from other geographical areas (6, 11, 12, 14). Type IV and type VI strains were not detected, as strains of these CPS types occur rarely (6, 11, 12, 14). Type VIII strains, frequently seen in Japan (17) and recently also in other areas (4, 8, 12), were not detected in the Zimbabwean
a large proportion of the isolates were type III strains, which
population, as only 121 isolates were serotyped. It is important that
existence of GBS of these CPS types in the Zimbabwean pop-
strain collection. However, this result does not exclude the
existence of GBS of these CPS types in the Zimbabwean pop-

Serovariants and serovariant markers. GBS strains of all of
the CPS types can be divided into subtypes or serovariants
on the basis of surface-anchored and strain-variable proteins.
In this study, we chose to search by PCR for the genes encod-
ing the proteins C_β, C_α, Alp1, Alp2, Alp3, R4/Rib, and Alp4.
Testing for R3 was MAb based. The testing resulted in the
division of the 121 GBS strains from Zimbabwe into 25 sero-
variants, with the highest number of variants occurring among
isolates of the CPS types Ia and V, which each included 6
variants (Table 1). Alp1 (epsilon) predominated among the
type Ia strains, the C_α-C_β combination predominated among
the type Ib and type II strains, and R4/Rib predominated
among the type III strains, essentially similar to earlier findings
for Zimbabwean GBS (23) and GBS from other areas (5, 11,
14). alp4, encoding Alp4, was not detected except in the re-
ference strain 9828, in which Alp4 was originally identified (14).
Distinctive features of the Zimbabwean GBS were related to
the CPS type V strains and concerned the R3 protein and the
genes alp3, encoding Alp3, and rib, encoding the R4/Rib pro-
gen. The gene alp2, encoding Alp2, was not detected in this
strain collection, but this gene and its product occur rarely (14,
18). Studies from other geographical areas have demonstrated a high prevalence of the gene alp3 or its product, Alp3, among
type V GBS, with up to 92% of type V strains carrying this
marker (14). Of 33 invasive type V GBS strains isolated in
Norway during the period from January to October 2007, 26
strains (78.8%) possessed alp3 (R. V. Lyng, personal com-
munication). Only 2 (6.9%) of the 29 Zimbabwean type V GBS
strains and only 6 (5.0%) of the strains in the whole collection
possessed alp3 (Table 1). Another feature of the Zimbabwean
type V strains was a comparatively high frequency (37.9%) of
rib possession. The rib gene was detected in only 1 of 38
Australasian type V GBS strains (14), and R4/Rib expression
in type V strains is considered to be rare (18). Also, recent
observations (21, 22) have generated suspicion that R4/Rib
expression by type V isolates may be overestimated when test-
ing is performed by means of antibody-based methods. This
question is due to the strong immunological cross-reactivity
between R4/Rib and Alp3 and the fact that the latter protein
is frequently expressed by type V strains from many geographical
areas (2, 11, 16, 18). Alp3 itself probably has no protein-
specific antigenic determinant, only determinants shared with
other Alps (21), although the encoding gene (alp3) possesses at
least one gene-specific primer binding site (14). Thus, available
information on R4/Rib expression by type V GBS may not be
reliable due to the disturbing existence of cross-reacting anti-
bodies.

A striking feature of the Zimbabwean type V GBS was their
frequent expression of the R3 protein, which was detected in
22 (75.9%) of 29 isolates, results which matched the findings
for isolates collected a few years earlier (23). R3 was the third
most common of the protein markers tested in this study (Ta-
ble 2) and was almost restricted to the CPS type V GBS. Only
four (4.3%) of the non-type V isolates expressed R3. These
results show that R3, not Alp3, is a predominating surface-
anchored protein in Zimbabwean type V GBS. It is possible
that this is the case in larger areas of southern Africa, but this
possibility remains to be verified. Although R3 was defined in
1972 (31), it has mostly been neglected in GBS serotyping,
though not entirely. The protein was not detected previously
among 131 U.S. strains (10). It was expressed by 6.5% of GBS
isolates from Norway (15). In the earlier study of GBS from
Zimbabwe, 24% of all isolates examined and 84% of the type

![FIG. 1. Western blots of sodium dodecyl sulfate lysates of whole
cells of GBS strains. Lysates from BM110 (III/R4/Rib) in lane 1, 335
(Ia/Alp1) in lane 2, ATCC 12403 (III/Alp2) in lane 3, ATCC 49447
(strain 10/84; V/R3) in lane 4, A909 (Ia/C_α, C_β) in lane 5, and 7271
(VII/Alp3) in lane 6 were probed with the anti-R3 MAb. Protein size
standards are shown to the left.]

TABLE 1. Distribution of CPS types and serovariants among 121
GBS carrier isolates from Zimbabwe

| CPS type (no. of strains) | Serovariant | No. of strains | % of strains |
|-------------------------|-------------|----------------|-------------|
| Ia (19; 15.7)           | Ia          | 2              | 10.5        |
|                         | Ia/Alp1     | 12             | 63.2        |
|                         | Ia/C_α, C_β | 1              | 5.3         |
|                         | Ia/Alp1, Alp3 | 1            | 5.3         |
|                         | Ia/C_β, Alp1 | 1             | 5.3         |
|                         | Ia/Alp1, R3  | 2              | 10.5        |
| Ib (14; 11.6)           | Ib/C_α      | 1              | 7.1         |
|                         | Ib/C_β      | 1              | 7.1         |
|                         | Ib/Alp1, R3 | 1              | 7.1         |
|                         | Ib/C_α, C_β | 11             | 78.6        |
| II (10; 8.3)            | II/Alp1     | 2              | 20.0        |
|                         | II/R4/Rib   | 3              | 30.0        |
|                         | II/C_α, C_β | 5              | 50.0        |
| III (47; 38.8)          | III         | 5              | 10.6        |
|                         | III/R4/Rib  | 40             | 85.1        |
|                         | III/Alp3, R4/Rib | 1 | 2.1        |
|                         | III/Alp3    | 1              | 2.1         |
| V (29; 24.0)            | V/R3        | 2              | 6.9         |
|                         | V/C_α, R3   | 6              | 20.7        |
|                         | V/R3, R4/Rib| 6              | 20.7        |
|                         | V/Alp1, R3  | 8              | 27.6        |
|                         | V/Alp3      | 2              | 6.9         |
|                         | V/R4/Rib    | 5              | 17.2        |
| NT (2; 1.7)             | NT/Alp3     | 1              | 50.0        |
|                         | NT/R3, R4/Rib | 1          | 50.0        |

* Calculated as the percentage of the total number of strains tested.
* Calculated as the percentage of isolates within each CPS type.
V strains in that collection expressed R3 (23), findings which match the results of the present study.

R3 presented with multiple bands on Western blots (Fig. 1 and 2) and with little strain variation in the molecular mass of the protein (Fig. 2). Whether the ladder-like pattern was due to a repetitive structure or was caused by the degradation of the protein, such as the hydrolysis of acid-labile bonds in the molecule (30), awaits clarification. We did not record immunological cross-reactivity between R3 and Cβ or Alp family members (Fig. 1), even when polyclonal anti-R3 was used for probing to confirm MAb-based results (data not shown). Alp family proteins have chimeric sequences, which explain why these proteins show immunological cross-reactivity (16, 18). The unique immunological specificity of R3 should accord with the unique sequence of this protein which, as yet, has not been analyzed by sequencing. A notable feature of R3 revealed in this study was its expression by strains which possessed one or another of the Alp family genes. In fact, 24 of the 26 R3-positive isolates showed combined R3 expression and Alp gene possession. On the other hand, only two isolates possessed more than one of the Alp genes, probably because Alp proteins are encoded by allelic genes (16, 18) and the gene encoding the R3 protein is not allelic, emphasizing its uniqueness. It is a possibility that the combined R3-Alp expression imparts advantages to GBS, for instance, in relation to pathogenic potential, notably since the combination occurred with particularly high frequency (69%) in type V strains, which have emerged as important pathogens in serious GBS infections (3, 6, 11, 13).

A protein called Fbs (type V group B surface protein), described in 1999 (2), was isolated from strain 10/84, the R3 reference strain used in the present study. Fbs had a molecular mass of ~110 kDa, close to that of the protein targeted by the R3 MAb (Fig. 1 and 2); was susceptible to trypsin and pepsin digestion, similar to the target of our R3 MAb, as shown in an earlier study (15); occurred frequently in type V strains and rarely in strains of other CPS types, similar to the R3 MAb target in the Zimbabwean GBS (Table 1); and was immunologically unrelated to other surface-anchored GBS proteins, also similar to the R3 MAb target (Fig. 1). Although Fbs failed to generate multiple bands upon Western blotting (2), unlike the MAB target (Fig. 1 and Fig. 2), the available data suggest that Fbs and the R3 MAb target may be identical. It is important that rabbit anti-Fbs antibodies were protective in an experimental model (2), which in the case of the two proteins’ being identical, means that R3 is a target of protective antibodies.

**Features of amplicons.** The sizes of the amplicons generated from the various cps or protein antigen genes were nearly identical for each particular gene, consistent with the stability of the sequences in the amplified stretches of these genes, except for the bac amplicons. The bac gene, which encodes Cβ, gave rise to amplicons with some size variation. Since Cβ possesses at least one stretch which varies in size (1), we sequenced the PCR products from two different isolates, amplicons of 647 and 632 bp. However, both amplicons had a sequence identical to that of the corresponding stretch of the sequenced gene (bac) encoding Cβ (GenBank accession no. X59771). We have no explanation for the size variation of the bac amplicons.

**Comments.** This study has shown that GBS from Zimbabwe were essentially similar to GBS from other geographical areas with respect to CPS types. Strains of the CPS types Ia, Ib, II, III, and V and NT strains were detected. As expected, for the CPS types Ia, Ib, II, and III, results for the genes encoding the strain-variable and surface-anchored Alp family proteins and for bac, encoding the Cβ protein, were also recorded. On the other hand, serovariant protein markers of the CPS type V strains of Zimbabwean origin differed considerably from those reported for type V GBS from Western countries. As many as 38% of the Zimbabwean type V strains contained rib, encoding the R4/Rib protein, otherwise known to be expressed rarely by CPS type V strains (18). The R3 protein was expressed by 75.8% of the Zimbabwean type V strains, while only 6.8% of the strains possessed alp3, which usually predominates among type V GBS. It seems that in the type V isolates from Zimbabwe, the R3 protein occupied the position and possibly the biological function held by Alp3 in type V strains from other areas. It is tempting to speculate whether this reflects evolutionary differences among lineages of GBS carried by humans which for thousands of years have lived in separated geographical areas. Comparative genome sequencing of representative type V strains from Zimbabwe and from other areas should be of great interest.

The development of a GBS protein vaccine has been an objective for a long time. Our results show that one or two of the three most prevalent proteins, R4/Rib, Alp1 (epsilon), and R3, were present in 75.2% of the Zimbabwean GBS strains tested. Generally, surface-anchored and strain-variable GBS proteins possess protective epitopes, as evidenced by experimental models (9, 18, 26, 28). Thus, it seems possible that if

| Protein | No. (%) of strains | Two most favored CPS type associations |
|---------|-------------------|---------------------------------------|
| R4/Rib  | 56 (46.3)         | III and V                             |
| Alp1    | 27 (22.3)         | Ia and V                              |
| R3      | 26 (21.5)         | V and Ia                              |
| Cβ      | 24 (19.8)         | Ib and II                             |
| Alp3    | 19 (15.7)         | Ib and II                             |
|         | 6 (5.0)           | V and Ia                              |

**Table 2. Frequency of occurrence of each of the protein markers studied among 121 GBS carrier isolates from Zimbabwe and most favored CPS type associations.**

**FIG. 2.** Western blots of sodium dodecyl sulfate lysates of whole cells of five Zimbabwean GBS strains positive for R3 expression in dot blotting (lanes 1 to 5) and of the R3 reference strain ATCC 49447 (strain 10/84; V/R3) (lane 6), probed with the anti-R3 MAb. Protein size standards are shown to the left.
introduced in Zimbabwe, a GBS vaccine which includes these three proteins may enhance resistance against infection with a large proportion of GBS strains. Also, it seems likely that a vaccine based on these proteins may result in increased protection against invasive Co- and Alp3-expressing GBS isolates due to Alp1-Cox cross-reactivity and R4/Rib-Alp3 cross-reactivity (2, 22, 28), offering the prospect of enhanced resistance against virtually all GBS variants found in this study. Such a vaccine also may induce protection against group A streptococcal variants which express the R28 (Alp3) protein (27, 28). The isolates under investigation were carrier strains. The serotype and serovariant distribution of invasive isolates from Zimbabwe may be different, as found by serotyping (11) or multilocus sequence typing (12) for GBS from other areas. Such data on isolates from Zimbabwe are not known but would be important. In South Africa, CPS type III strains predominate as the cause of early- and late-onset neonatal disease, while for instance, type V strains play a less prominent role (19). This pattern is different from the role of CPS type V strains in invasive GBS disease in many other areas (3, 6, 11, 13).

The results of the present study have substantiated that R3 is an important serovariant marker, at least in GBS isolated from humans in certain geographical areas. Accordingly, this protein may be important in the pathogenesis of GBS disease caused by R3-expressing strains, and as R3 may be a vaccine candidate, R3 protein detection should be pursued actively in GBS typing.

ACKNOWLEDGMENTS

We are grateful to R. V. Lyng, P. Masunga, P. Madekafumba, and E. Meque for their technical assistance. We are also grateful for the support by the Norwegian Quota Program for students from developing countries and Central and Eastern Europe.

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