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Short communication

Virulence characteristics of genetically related isolates of group B streptococci from bovines and humans

A.B.A. Corrêa, M.A. Américo, I.C.M. Oliveira, L.G. Silva, M.C. de Mattos, A.M.M. Ferreira, J.N.S.S. Couceiro, S.E.L. Fracalanzza *, L.C. Benchetrit

Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, RJ 21941-590, Brazil

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ABSTRACT

The present study had the objective of evaluating the pathogenic potential of the genetically related strains of *Streptococcus agalactiae* no. 80427 (human origin) and no. 87159 (bovine origin), and comparing the results with two other strains isolated from bovine mastitis (no. 87244) and invasive human infection (no. 90356), with no genetic or epidemiologic relationship between them or with the first 2 isolates. Virulence genes *hylB* (hyaluronidase) and *lmb* (laminin-binding protein) were detected in the 4 strains, and genes *bac* (beta protein) and *bca* (alpha protein) were only detected in human strains. The protein profile obtained using SDS-PAGE did not indicate any differences between the 4 strains. No significant difference was detected between human and bovine strains in the assays of adherence to and invasion of 16HBe cells, as well as in the resistance assay for intracellular bacterial survival in macrophages. However, the strain 87159 exhibited a greater survival in the killing test with whole human blood and was more virulent in newborn mice than the 80427 strain. The strain 87244 was not virulent in mice. These data suggest that isolates of human and bovine origins may express similar virulence attributes, leading to a possible, however limited, dissemination.

1. Introduction

Group B *Streptococcus* (GBS) was initially described in 1887 as an animal pathogen that caused bovine mastitis (Ross, 1978), being considered of great importance to veterinary medicine ever since, significantly affecting the economic activity related to the milk and dairy products industry (Bradley, 2002). Later on, GBS was frequently associated with a large variety of serious human infections, among which sepsis and meningitis in neonates (Eickhoff et al., 1964; Baltimore, 2007). Therefore, the study of virulence of animal and human strains, as well as assays to determine their ability to colonize and cause diseases in the human host, are of great interest for the understanding of GBS epidemiology, along with possible interspecies transmission and colonization (Sukhananand et al., 2005; Héry-Arnaud et al., 2007).

For this reason, GBS has stimulated extensive investigations in basic research using phenotypic and genotypic techniques for strain classification, as well as the study of the genetic relationship between human- and bovine-isolated strains. Many works have suggested a relationship between human and the bovine niches (Poutrel and Dore, 1985; Jensen and AArestrup, 1996; Martinez et al., 2000; Héry-Arnaud et al., 2007). Recently, a genetic relatedness between a human and a bovine strain was established by PFGE (pulsed field gel electrophoresis), ribotyping and MLST (multi locus sequence typing) in a study conducted in Brazil (Oliveira et al., 2006).

* Corresponding author at: Instituto de Microbiologia Prof. Paulo de Góes, Centro de Ciências da Saúde - Bloco I. Av. Carlos Chagas Filho 372, Cidade Universitária - Ilha do Fundão - Rio de Janeiro, RJ 21941-590, Brazil. Tel.: +55 21 2562 6504.
E-mail address: fracalanzza@micro.ufrj.br (S.E.L. Fracalanzza).

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In a previous study, we demonstrated the genetic relationship between one human strain and one bovine isolate from Brazil (Oliveira et al., 2006), but we did not investigate their virulence properties. The objective of the present work was to evaluate the virulence traits of 2 genetically related GBS strains of human and bovine origins in relation to 2 other genetically unrelated strains.

2. Methodology

Bacterial strains and growth conditions. The 4 GBS strains used in this work were previously described by Oliveira et al. (2006). Two strains were of serotype V and genetically related: 80427 (human cervix, Rio de Janeiro 1980, PFGE P1 and Sequence Type, ST, 26 profile) and 87159 (bovine mastitis, São Paulo 1987, PFGE A2 and ST 256 profile). Profiles P1 and A2 were indistinguishable. In addition, two genetically unrelated strains were used: 87244 (bovine mastitis, Rio de Janeiro 1987, serotype II, PFGE B profile) and 90356 (cerebrospinal fluid, Rio de Janeiro 1990, serotype III, PFGE G2 profile). The 4 strains were cultivated at 37 °C in Todd–Hewitt broth (THB; Difco) until a DO_{540} of 0.4, and the sediments was washed and resuspended in an appropriate medium or buffer and served for the starting materials for the experiments.

Polymerase chain reaction for the detection of virulence genes. DNA extraction was performed as described by Sambrook et al. (1989). The polymerase chain reaction was performed in a GeneAmp PCR System 2400 (Applied BioSystems), as follows: 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min; and 72 °C for 4 min. The amplified products were stained with ethidium bromide after agarose gel electrophoresis. The 100 bp lambda ladder kit (Invitrogen) was used as DNA size standard. Detection of genes associated with GBS virulence – *hylB* (hyaluronidase), *bac* (beta protein), *bca* (alpha protein) and *lmb* (laminin-binding protein) – was carried out as described earlier (Corrêa et al., 2009). Furthermore, detection of the housekeeping genes *stp* (serine/threonine phosphatase) or *stk* (serine/threonine kinase) was used as a positive control in the reactions. The initiators for the *hylB*, *bac* and *bca* genes were previously described (Corrêa et al., 2009). Other initiators used were 5′-CCGTCTGTAAATGATGTGGC-3′ and 5′-GAAATACCCGAGATACCAAG-3′ (*lmb*); 5′-GATATTGGGCAACGTCGTTC-3′ and 5′-GATACTCTCCTTGACGAACG-3′ (*stp*); and 5′-GACAACTTATGAGATGGGAC-3′ and 5′-CGACTGTGCTAAGATTGTAC-3′ (*stk*).

Adherence and invasion assays. Assays regarding adherence to and invasion of 16HBe bronchial pulmonary epithelium cells were performed using the protocol described by Schubert et al. (2004). The 16HBe cells were propagated in a 199 tissue culture medium (Gibco BRL) with 10% fetal bovine serum (FBS; Gibco BRL) at 37 °C and atmosphere of 5% CO_2, and then transferred to 24-well tissue culture plates (10^5 cells per well). After overnight culture in a 199 medium supplemented with 10% FBS, the 16HBe cells were infected with 10^6 CFU in a 199 medium and incubated at 37 °C for 2 h. Subsequently, the infected cells were washed three times with PBS. The number of adherent bacteria was determined after disruption of eukaryotic cells with triton X-100 (0.01%; v/v) and subsequent determination of the number of CFU through plating of the appropriate lysate solutions in sheep blood agar. Intracellular bacteria were quantified after additional incubation of infected cells for 2 h in a 199 medium containing penicillin G (10 U/ml) and gentamicin (100 μg/ml). After three washes with PBS, epithelial cells were disrupted and the number of intracellular bacteria was determined.

Bacterial intracellular survival test in macrophages. Survival within murine macrophages was evaluated using the protocol described by Poyart et al. (2003). Eight-week-old Balb/c mice were submitted to a 1 mL intraperitoneal injection of a 10% sodium thioglycolate solution. After 96 h, cells from the peritoneal exudate were collected through washing with 199 medium containing 10% FBS and penicillin G (10 U/ml). Cells with macrophage-like characteristics were counted in a hemacytometer and plated in 24-well culture plates. After 1 h of incubation at 37 °C in the presence of 5% CO_2, the non-adherent cells were removed through washing with medium, and the adherent cells were once again incubated. After 24 h, the confluent monolayers were infected with $(1–5) \times 10^6$ CFU in a 199 culture medium supplemented with 10% FBS. The mixture was incubated during 15 min at 4 °C for adherence, and during 30 min at 37 °C for bacterial invasion. After this incubation (time 0 of the assay), penicillin G (10 U/ml) and gentamicin (100 μg/ml) were added to kill extracellular bacteria. In order to determine the number of intracellular streptococci at different times of infection, supernatants were removed, macrophages were washed three times with PBS, followed by disruption with Triton X-100 (0.1%; v/v). Serial dilutions of the lysate of each well were plated onto sheep blood agar. The number of CFU was determined after 24 h of incubation at 37 °C.

Killing assay in whole human blood. The assay was conducted according to the protocol described by Liu et al. (2004). An inoculum of 10^4 CFU in 0.3 mL of PBS was mixed with 0.6 mL of fresh human blood collected in a heparin-treated tube and incubated under agitation for 3 h at 37 °C. Thereafter, dilutions were plated onto sheep blood agar to determine the number of CFU.

In vivo virulence studies. Newborn Balb/c mice (4 days old; 11–34 animals according to the strain tested) were used. Animal groups were inoculated intraperitoneally with 0.01 mL of strains in physiological solution (10^4 CFU). The animals were isolated in boxes with their mothers during the study period and monitored daily for survival. These experiments were conducted according to the Animal Care Committee Policy of the Federal University of Rio de Janeiro.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Bacterial strains were resuspended in TE buffer (Tris–HCl 10 mM pH 8.0; disodium EDTA 1 mM; pH 8.0), sonicated under maximum output for 4.5 min in an Ultrasonic High Intensity Processor (Sigma), and centrifuged at $275 \times g$ for 20 min. SDS-PAGE was performed according to Laemmli (1970). After electrophoresis, proteins were stained with Coomassie Brilliant Blue (Sigma).

Statistical analysis. Differences in mice mortality after inoculation with GBS were analyzed through χ^2 tests.