Lack of Virulence of Bovine Type III *Streptococcus agalactiae* Strains for Mice Correlates with Reduced In Vitro Production of Extracellular Type-Specific Antigen

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Abstract. Six strains of type III *S. agalactiae* isolated from milk samples from cases of bovine mastitis were examined for in vitro production of three potential extracellular virulence factors: neuraminidase, protease, and extracellular type-specific antigen. Virulence in mice, expressed as LD₅₀ values, was examined for these six strains to determine if a relationship existed between the in vitro production of any of the three extracellular products and mouse lethality. Only in vitro production of extracellular type-specific antigen showed a correlation with virulence of these organisms in the mouse model. All six bovine strains were relatively avirulent in the mouse while producing reduced levels in vitro of extracellular type-specific antigen as compared to nine human isolates. The bovine *S. agalactiae* strains were an average of 538-fold less virulent for the mouse than were the high type-specific antigen producers isolated from human sources.

*Streptococcus agalactiae* is the cause of approximately 30% of the mastitis in dairy cows in the United States and this has been estimated to result in at least a $350,000,000 a year loss to dairymen [3]. Currently available eradication methods for *S. agalactiae* are time consuming and labor intensive. Accordingly, complete eradication of this organism in bovine mastitis has not been achieved [16]. There is an obvious need for more basic research on *S. agalactiae* which might be useful in the formation of more effective eradication measures in the future. In addition, human neonatal infections caused by *S. agalactiae* represent an important public health problem in this country and the reasons for the emergence of this organism as a significant neonatal pathogen within the last twenty years are obscure. There is considerable controversy concerning the natural reservoir of human and bovine strains of *S. agalactiae* and few consistent differences between human and bovine isolates have been documented [7,8]. Indeed, in a comparative study [10] to characterize human and bovine *S. agalactiae* isolates by means of a bactericidal assay, it was concluded that there were no principle differences within the mouse virulent (human) or mouse avirulent (bovine) strains. Also examined, in the same study, were the bactericidal effects of human and bovine white blood cells and sera. The origin of the strain was taken into consideration and once again no principal differences were found. It was stated [10] that “strains isolated from man and cattle are identical and therefore may be a cause of zoonotic diseases.” Therefore, we feel that this relationship between bovine and human isolates of *S. agalactiae* should be investigated not only for economic reasons, but because these bovine strains represent a possible potential public health threat to newborn infants.

We have recently studied several of the potential extracellular virulence factors elaborated by type III *S. agalactiae* in vitro [6]. In that report, we examined twelve human *S. agalactiae* isolates for their in vitro production of these potential extracellular virulence factors: extracellular type specific antigen (ETSA), protease, and neuraminidase. These extracellular products were examined because correlations with virulence have been demonstrated in other bacteria between protease [9,11,17] and neuraminidase production [12,15], as well as between the severity of infection and circulating ETSA [18]. The results showed that type III human

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isolates of *S. agalactiae* that produced elevated levels of ETSA tended to be significantly more virulent for the mouse than did type III human isolates that produced reduced levels of ETSA [6]. In this report, six bovine type III strains of *S. agalactiae* isolated from milk samples from bovine mastitis for the in vitro production of these three potential extracellular virulence factors were studied. All produced low levels of ETSA and were relatively avirulent in a mouse model.

**Materials and Methods**

**Bacterial strains and experimental animals.** Serotype III *Streptococcus agalactiae* strains 230, 261, 494, 495, 498, and 501 were isolated from milk samples of cows with mastitis and were supplied by J. S. McDonald, National Animal Disease Center, Ames, Iowa. Mouse lethality tests were performed as previously described [6] employing 6-week-old ICR mice (Simonsen Laboratories, Gilroy, California). The organisms were grown in Todd-Hewitt broth and injected intraperitoneally (i.p.). The quantitation of in vivo extracellular type specific antigen (ETSA) in sera from infected mice was done by rocket immunoelectrophoresis (RIE) [6]. LD_{50} values for the various strains were calculated by the method of Reed and Muench [20].

**Purification and quantitation of extracellular products.** The organisms were grown in a chemically defined medium (FMC) at 37°C and neuraminidase and protease were quantitated [6]. For quantitation of ETSA, the supernatant fluid from a liter culture of each of the strains of type III *S. agalactiae*, grown in FMC [4] to the stationary phase, was chromatographed in 10 mM sodium acetate (pH 6.5) on a Sepharose 4B column (2.5 x 90 cm) to separate the group B antigen and ETSA. The eluting fractions were collected (4 ml/fraction) and assayed for sialic acid content [1] as well as for type III specificity by the capillary precipitin reaction. The peaks containing ETSA were then pooled and the amount of ETSA produced by each of the bovine isolates was determined by RIE [6].

**Statistics.** Significance of differences between LD_{50} values of high human and low bovine ETSA producers were determined by standard deviation methods [13]. Unpaired Student’s *t* test was used to compare assayed levels of ETSA for significant differences [22].

**Results**

**Purification of Streptococcus agalactiae bovine type III extracellular type specific antigen (ETSA).** The elution profiles of the six bovine strains (by sialic acid determination) were remarkably similar to the Sepharose 4B elution profiles of type III *Streptococcus agalactiae* human isolates [5,6] (data not shown). The pooled ETSA of each of the six bovine strains produced a strong capillary precipitin reaction with type III rabbit antiserum produced against a human type III *S. agalactiae* isolate (*S. agalactiae* 110). This was also the case in Ouchterlony immunodiffusion analyses when these antigens were examined against type III specific antiserum. As can be seen in Fig. 1A, the Sepharose 4B chromatographically purified ETSA from all six bovine strains showed lines of identity among themselves, and with the ETSA of a human strain (*S. agalactiae* 110) when examined by immunodiffusion against type III specific antisera (Fig. 1B, 1C). In addition, the ETSA from bovine strain 501 was further purified by diethylaminoethyl (DEAE) cellulose chromatography followed again by Sepharose 4B gel filtration chromatography [4]. This material was subjected to gas liquid chromatography [21] after acid hydrolysis (2 N HCl at 100°C for 4 h). This analysis revealed the presence of glucose, galactose, and glucosamine and these compounds, as well as sialic acid, are the components that have been shown [4,5] to comprise the ETSA of human *S. agalactiae* isolates.

**Quantitation of extracellular neuraminidase and protease.** The supernatants from 50 ml and 6 liter cultures [6] of the six bovine strains were assayed for neuraminidase and protease activity, respectively (Table 1), in order to see if enzymatic activity correlated with the amount of ETSA produced. Three of the strains tested, 261, 498, and 501, were extremely high producers of neuraminidase (greater...