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Detection of enzyme activities and their relation to serotypes of bovine and human group B streptococci

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Enzymatic properties of group B streptococci (GBS) serotypes from bovine milk and human routine vaginal specimens were investigated. Out of the 56 human and 66 bovine GBS, 35 and 30 could be classified serologically by a co-agglutination test with type-specific antisera, respectively. Hyaluronidase (HYAL), streptokinase (SK) and protease activities were detected using culture media. HYAL activity was observed mostly in typable human GBS, and serotypes Ia, Ic and II comprised 77.3 % of the typable strains producing HYAL. Bovine GBS serotypes II, III and VII comprised 87.5 % of typable bovine strains exhibiting HYAL activity. SK activity was detected only in three human GBS. Human GBS serotypes Ia, Ic, II, III, VII and almost all typable bovine GBS strains showed protease activity. β-D-glucosidase activity was frequently observed in human GBS, whereas *N*-acetyl-β-D-glucosaminidase activity was mostly detected in non-typable GBS from humans. These results indicate that different GBS serotypes could vary in their virulence properties, and bovine and human GBS isolates could not be differentiated by their enzyme activities. Use of the culture media appeared to be a simple-to-apply and useful method for the detection of extracellular enzyme activity such as HYAL, protease and SK.

INTRODUCTION

Group B streptococci (GBS) are a well-known causative agent of contagious bovine mastitis and serious human perinatal infections. GBS possess a variety of potential virulence factors on the bacterial surface, including capsular polysaccharides and c (α/β) protein with distinct structural and antigenic types or serine protease and C5a peptidase (Spellerberg, 2000; Yildirim *et al.*, 2002). Capsular components may assist in enhancing adaptation of the organism to various tissues within the host and a sialylated GBS capsule protects GBS by interference with opsonophagocytosis. Fibronectin-binding protein possesses a peptidase domain that specifically cleaves human complement component C5a, ablating its neutrophil chemo-attractant property. Also, a related GBS cell surface protease, CspA, targets host fibrinogen, producing adherent fibrin-like cleavage products that coat the bacterial surface and interfere with opsonophagocytic clearance (Doran & Nizet, 2004).

GBS are serologically classified into at least nine capsular polysaccharide antigen types (Ia, Ib, II, III, IV, V, VI, VII and VIII) and three protein antigen types (Ic, R and X) (Jelínkoá, 1977; Spellerberg, 2000). Serotyping based on the capsular polysaccharide and protein antigen of the cell wall gives more information about common or different characteristics between bovine and human GBS as well as their epidemiological distribution (Devriese, 1991; Ekin & Gurturk, 2006).

Several different bacterial products, such as extracellular enzymes and toxins, are also considered to be important in pathogenesis of streptococcal infections. High-level enzyme production is frequently found in most virulent strains of GBS. One of them is an extracellular hyaluronidase (HYAL; hyaluronate lyase) which is secreted by many clinical isolates of GBS (Lin *et al.*, 1994). Hyaluronic acid is a major component of the connective tissue and HYAL cleaves the glycosidic bond between *N*-acetyl-β-D-glucosamine and *N*-acetyl-β-D-glucuronic acid residues and is regarded as a spreading factor (Hynes & Walton, 2000; Spellerberg, 2000). Streptokinase (SK; fibrinolysin) is a protein secreted by several species of streptococci and may play an important role in virulence by facilitating invasion of bacteria in host tissues. The enzyme activates plasminogen hydrolytically through bond cleavage to produce plasmin. During streptococcal infections, extra production of plasmin may breakdown unwanted blood clots especially pulmonary embolism in the lung (Mundada *et al.*, 2003).

Abbreviations: GBA, β-glucosidase; GBS, group B streptococci; GUSB, β-D-glucuronidase; HYAL, hyaluronidase; NAGase, *N*-acetyl-β-D-glucosaminidase; NT, non-typable; SK, streptokinase; ST, serotypable.

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Extracellular protease activity as detected by casein hydrolysis was also found to be an essential characteristic of GBS from bovine milk for the acquisition of nutrients through milk degradation, leading to survival of bacteria inside the udder (Duarte *et al.*, 2004). Detection of caseinase activity using culture media in human GBS and *Pseudomonas aeruginosa* was reported previously (Straus *et al.*, 1980; Janda & Bottone, 1981).

N-acetyl-β-D-glucosaminidase (NAGase) cleaves all non-reducing β-linked *N*-acetylglucosamine residues in chitobiose and degrades mucopolysaccharides and glycoproteins. It was found to be responsible for mucolipidosis and various inflammatory disorders of muscle and connective tissue (Hussain *et al.*, 1992; Clarke *et al.*, 1995). β-glucosidase (GBA) catalyses the hydrolysis of terminal non-reducing residues in β-D-glucosides through cleaving β-D-glucosidic linkages in glucose-substituted molecules and plays an important role in fundamental biological processes (Jeng *et al.*, 2011). Streptococcal β-D-glucuronidase (GUSB) is an intracellular substance that catalyses hydrolysis of β-D-glucuronic acid residues from the non-reducing end of mucopolysaccharides (Jain *et al.*, 1996).

There is limited information on the HYAL, SK and protease production of GBS from bovine and human sources detected using culture media. Recent studies are based on the detection of HYAL-related genes with different genome size and provide information from a limited number of GBS isolates from cattle and humans (Yildirim *et al.*, 2002; Sukhnanand *et al.*, 2005; Corrêa *et al.*, 2010). Since the first report on the protease production of human GBS serotype III was published by Straus *et al.* (1980), no further findings on SK and protease activities of GBS from both sources and their relationship with serotypes have been reported until now.

In a previous study, we demonstrated a biochemical and serological relationship between human and GBS strains isolated in Van (Ekin & Gurturk, 2006), but we did not investigate how extracellular enzyme activity contributed to virulence. In the present study, HYAL, SK, protease, NAGase, GBA and GUSB enzyme activities of GBS strains from bovine and human sources and their relationship with serotypes are discussed.

METHODS

Bacterial isolates. In this work, a total of 122 GBS strains, 66 from lactating cows' milk originating from widely different locations around Van Lake basin, eastern Turkey, and 56 from women's routine vaginal specimens obtained from maternity clinics located in Istanbul and Van were used. Fifty bovine and 21 human GBS strains used in this study were described previously by Ekin & Gurturk (2006). The remaining 16 bovine isolates from Van and 35 human isolates from Istanbul were identified by haemolytic activity, CAMP reaction, aesculin and sodium hippurate hydrolysis and serogrouping with GBS specific antiserum as described previously (Facklam, 2002).

Reference strains. Reference strains of *Streptococcus agalactiae* serotypes Ia (090), Ib (H 36 B), Ic (A 909), II (18 RS 21), III (6313), IV (3139), V (SS 1169), VII (7271), VIII (JM9 130013), R (25/60 Compton) and X (24/60 Compton) and *Staphylococcus aureus* Cowan I (NCTC 8530) were kindly provided by Professor Dr Christoph Lämmler (Institut für Pharmakologie und Toxikologie Fachbereich Veterinärmedizin der Justus Liebig Universität, Giessen, Germany).

Serogrouping. Serogrouping was performed with streptococcal grouping reagent B rapid latex agglutination test according to the manufacturer's instructions (Oxoid). The isolates were grown at 37 °C overnight on blood agar base (Merck) with 5 % (v/v) sheep blood.

Serotyping. Type-specific antisera were prepared in rabbits by the inoculation of heat-killed suspensions (60 °C for 30–60 min) of each reference GBS serotype, Ia, Ib, Ic, II, III, IV, V, VII, VIII, R and X cultures. Mono-specific antisera were obtained by adsorption with each cross-reactive GBS serotype culture (Jelinkova, 1977; Ekin & Gurturk, 2006). Serological typing was performed by the co-agglutination method using staphylococcal protein A coated mono-specific antibodies according to the recommendations of Christensen *et al.* (1973). Antigens were prepared using a hot HCl extraction assay (Jelinkova, 1977).

HYAL assay. GBS isolates were examined for HYAL production on hyaluronidase test medium [brain–heart infusion broth (Oxoid) with 1 % Noble agar (Difco)] containing 0.4 mg ml^{-1} hyaluronic acid potassium salt from human umbilical cords (Fluka) and 10 mg BSA fraction V ml^{-1} (Sigma) as described by Smith & Willett (1968). After overnight growth of spot inocula at 37 °C the test medium was developed by flooding with 2 mol glacial acetic acid l^{-1} (Merck) for precipitation of a hyaluronidate-BSA complex. Positive colonies were surrounded by clear zones on a turbid background.

SK assay. For the detection of SK activity, nutrient agar (Difco) containing 2.8 mg human fibrinogen fraction I type III ml^{-1} (Sigma) and 0.03 ml canine plasma was prepared as described by Janda & Bottone (1981). To detect the SK activity of GBS, five or more colonies from overnight GBS culture on tryptic soy agar (Oxoid) were spotted on the test medium. After overnight incubation at 37 °C, positive reactions were identified by clear zones surrounding colonies on a turbid background.

Protease assay. Protease activity was detected on brain–heart infusion agar (Oxoid) containing 15 mg skimmed milk powder ml^{-1} (Oxoid) (Sokol *et al.*, 1979). Overnight GBS culture was spotted on the test medium, and after 18–24 h of incubation at 37 °C, protease activity was determined by a clear zone around the spot colonies.

4-Methylumbelliferyl-conjugated substrates. NAGase, GBA and GUSB enzyme activities were detected using 4-methylumbelliferyl-conjugated substrates as described by Maddocks & Greenan (1975).

A stock solution of 15 µmol 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide l^{-1} (for NAGase; Sigma) was prepared in 0.2 ml *N,N*-dimethylformamide (Sigma), and stock solutions of 15 µmol 4-methylumbelliferyl-β-D-glucuronide hydrate l^{-1} (for GUSB; Sigma) and 4-methylumbelliferyl-β-D-glucopyranoside (for GBA; Fluka) were each prepared in 0.2 ml DMSO (Merck). The individual stock solutions were diluted to 10 ml in 0.2 mol acetate l^{-1} buffer (pH 5.2). The enzyme activity of GBS was determined by vigorously rubbing five or more colonies on filter paper (no. 1, Whatman) using a wooden stick and by adding a drop of substrate on the colonies. After incubation at 37 °C for 20 min, 50 µl 0.1 mol NaOH l^{-1} (Riedel-de Haën) was added to the spot on the paper and the methylumbelliferone fluorescence visualized under UV light (4 W, 366 nm; Merck) in a dark area. Light blue fluorescence was considered to be positive.

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Table 1. Comparison of enzyme activities with ST and non-typable (NT) GBS strains from bovine and human sources (%)

Source		*n*	HYAL	SK	Protease	NAGase	GBA	GUSB
Bovine	ST	30	8 (26.66)	0	21 (70.00)	16 (53.33)	10 (33.33)	14 (46.66)
	NT	36	5 (13.88)	0	24 (66.66)	18 (50.00)	12 (33.33)	15 (41.66)
	Total	66	13 (19.69)*	0	45 (68.18)†	34 (51.51)	22 (33.33)‡	29 (43.93)
Human	ST	35	22 (62.85)‡	1 (2.85)	14 (40.00)†	16 (45.71)[c]	24 (68.57)	18 (51.42)
	NT	21	3 (14.28)‡	2 (9.52)	14 (66.66)†	15 (71.42)[c]	15 (71.42)	15 (71.42)
	Total	56	25 (44.64)*	3 (5.35)	28 (50.00)†	31 (55.35)	39 (69.64)‡	33 (58.92)

*$P<0.01$.
†$P<0.05$.
‡$P<0.001$.

Statistical analysis. A statistical analysis was carried out using the Z-ratio test in the Minitab version 16 package program (accessed 15 January 2015) according to the Z-Ratio test.

RESULTS

According to serotyping of new isolates (16 bovine, 35 human) and combining with old cultures, 35 (62.5 %) human and 30 (45.5 %) bovine GBS isolates were evaluated as serotypable (ST) using mono-specific antisera, whereas 21 (37.5 %) human and 36 (54.5 %) bovine GBS isolates did not react with any type-specific antisera (Table 1). Bovine ST strains reacted with GBS serotype Ic (6), II (2), III (5), IV (1), V (1), VII (7), VIII (3), R (1), X (2) and III-Ic (2) type-specific antisera. Human ST strains were positive for GBS serotypes Ia (7), Ic (2), II (10), III (6), IV (2), VII (3), R (3), II-IV-X (1) and III-R (1) (Table 2).

HYAL activity was detected in 25 (44.6 %) human and 13 (19.7 %) bovine GBS. Three (14.3 %) non-typable (NT) human and five (13.9 %) NT bovine GBS also showed HYAL activity. Twenty-two (62.9 %) ST human GBS isolates were positive for HYAL, and serotypes Ia, Ic and II comprised 77.3 % of the ST human strains producing HYAL. Only one of the six human GBS type III isolates showed HYAL activity. Eight (26.7 %) ST bovine GBS had HYAL activity, and serotypes II, III and VII comprised 87.5 % of the typable bovine isolates showing HYAL activity (Tables 1 and 2).

Three (5.4 %) human GBS isolates, one serotype VII and two NT GBS, showed SK activities. SK activity could not be observed in bovine GBS isolates (Tables 1 and 2). Protease activity was detected in 45 (68.2 %) bovine GBS and 28 (50 %) human GBS isolates (Table 1). Fourteen (66.7 %) human NT and 24 (66.7 %) bovine NT GBS produced protease. Excepting bovine GBS serotype V,

Table 2. Distribution of enzyme activities in ST GBS isolates from bovine and human sources

Serotype	*n*		HYAL		SK		Protease		NAGase		GBA		GUSB	
	B	H	B	H	B	H	B	H	B	H	B	H	B	H
Ia	–	7	–	7	–	–	–	4	–	4	–	6	–	6
Ic	6	2	–	2	–	–	5	1	3	1	1	–	3	1
II	2	10	2	8	–	–	2	4	2	3	1	7	2	2
III	5	6	2	1	–	–	2	2	2	2	1	5	2	3
IV	1	2	–	1	–	–	1	–	1	2	1	2	–	2
V	1	–	–	–	–	–	–	–	–	–	–	–	–	–
VII	7	3	3	1	–	1	5	2	5	2	3	1	5	3
VIII	3	–	–	–	–	–	2	–	1	–	1	–	1	–
R	1	3	–	1	–	–	1	–	1	1	1	2	1	–
X	2	–	–	–	–	–	2	–	–	–	–	–	–	–
II-IV-X	–	1	–	1	–	–	–	1	–	1	–	1	–	1
III-R	–	1	–	–	–	–	–	–	–	–	–	–	–	–
III-Ic	2	–	1	–	–	–	1	–	1	–	1	–	–	–
NT	36	21	5	3	–	2	24	14	18	15	12	15	15	15
Total	66	56	13	25	–	3	45	28	34	31	22	39	29	33

B, bovine; H, human; NT, non-typable.

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two human GBS type IV and three human GBS type R isolates, protease activity could be detected in almost all bovine and human GBS serotypes at different rates.

In relation to enzyme activity detected using 4-methylumbelliferyl-conjugated substrates, NAGase, GBA and GUSB enzyme activities were detected in 31 (55.4 %), 39 (69.6 %) and 33 (58.9 %) human GBS and in 34 (51.5 %), 22 (33.3 %) and 29 (43.9 %) bovine GBS isolates, respectively. NAGase activity was mostly found in NT human GBS isolates, whereas the same enzyme activity could be detected in NT and ST bovine GBS isolates at an equal rate. Concerning GBA and GUSB enzyme activities, there was also no significant difference between NT and ST GBS strains from human and bovine sources (Table 1).

No enzyme activities were detected in bovine GBS serotype V and human GBS serotype III-R combinations. Two bovine GBS serotype X isolates showed only proteolytic activity.

DISCUSSION

Detection of extracellular enzyme activities of bacteria, such as HYAL, SK and protease could be easily performed *in vitro* in culture media as described previously (Smith & Willett, 1968; Sokol *et al.*, 1979; Janda & Bottone, 1981; Slifkin & Gil, 1983). Recently, many papers have also been published describing the detection of virulence genes of GBS related to molecules either located on the bacterial surface or secreted in the surrounding environment (Fernandez-Espla *et al.*, 2000; Spellerberg, 2000; Corrêa *et al.*, 2010). HYAL is a putative virulence factor that is involved in cleaving hyaluronic acid, a component of the extracellular matrix, in many tissues (Lin *et al.*, 1994).

A recent study (Sukhnanand *et al.*, 2005) reported that HYAL activity of human GBS isolates (28.8 %) was significantly lower than those of bovine isolates (53.8 %). In contrast, in the present study, HYAL activity of bovine isolates (19.7 %) was lower than that of human GBS isolates (44.6 %). There is little information on the HYAL activity of GBS serotypes. A previous study (Kjems *et al.*, 1980) also showed that 75 % of human ST (serotypes Ia, Ib, II, III) and 73 % of NT GBS isolates did produce HYAL. In the present study, HYAL activity of human ST isolates (62.9 %) was significantly higher ($P<0.01$) than that of human NT isolates (14.3 %). Also, human GBS serotypes Ia, Ic and II comprised 77.3 % of the typable human strains producing HYAL. On the other hand, serotypes II, III and VII were the predominant bovine isolates showing HYAL activity at different rates. Yildirim *et al.* (2002) also reported that hyaluronate lyase enzyme activity was detected in NT/X, Ia/X, III/Rib, Ib/c$\alpha\beta$ and NT GBS isolates from cattle and II/Rib, V/cα, II/cα, Ia, Ib/c$\alpha\beta$ and Ia/c$\alpha\beta$ GBS isolates from humans.

SK is a single-chain 414 aa protein secreted by β-haemolytic group A, C and G streptococci and is an efficient plasminogen activator which has been associated with the pathogenesis of post-streptococcal glomerulonephritis (Lähteenmäki *et al.*, 2001). There are no available reports on the SK activity of human or bovine GBS. However, in the present study, SK activity was detected in only 3 (5.4 %) human GBS isolates. All three SK-producing GBS strains did also produce protease, but not HYAL. Protease activity of GBS appeared to be independent of their SK activity since the majority of human GBS showing protease activity seemed not to produce SK.

Some of the proteases inactivate host-defence proteins, such as immunoglobulins, complement components, antimicrobial peptides, and others are required for intracellular survival in macrophages and for adherence as well as uptake into epithelial cells (Gaillot *et al.*, 2000; Lähteenmäki *et al.*, 2001). Duarte *et al.* (2004) reported that almost all GBS isolates recovered from bovine milk with clinical and subclinical mastitis showed protease activity detected using culture media. They emphasized that serotype III was the predominant GBS strain followed by serotypes II, Ia, Ib and VI. In the present study, protease activity detected by skimmed milk medium for casein hydrolysis was not found to be a characteristic in both bovine and human GBS. Our findings also showed that the protease activity could be detected in both NT and ST strains of GBS.

Several authors have proposed the detection of NAGase, GBA and GUSB enzymes for the characterization of GBS strains. Slifkin & Gil (1983) reported that all human clinical GBS isolates are able to produce GUSB but not GBA. Similar findings were reported for GBS strains isolated from clinical mastitis (Schaufuss *et al.*, 1986; Fang *et al.*, 1995). In our previous study (Ekin *et al.*, 2010), 80 %, 86.6 % and 80 % of human NT GBS isolates and 30 %, 40 % and 37.5 % of bovine NT GBS isolates were found to be positive for NAGase, GBA and GUSB, respectively. In this study, statistically significant ($P<0.001$) differences in GBA activity were determined between total bovine and human GBS isolates.

In conclusion, the use of culture media appeared to be a simple–to–apply and useful method for the detection of extracellular enzyme activity such as HYAL, protease and SK. HYAL production seemed to be characteristic of typable strains of human GBS. SK activity could not be detected in bovine GBS, whereas three human isolates were found to be positive in this study. Further studies using molecular genetic methods are necessary for characterizing protease and SK activity detected in culture media and their importance for the virulence of the GBS strains from bovine and human sources.

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