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ORIGINAL ARTICLE

Biotyping of Nontypeable Group B Streptococci

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SUMMARY

In this study the nontypeable Group B Streptococci (GBS) of bovine and human origin were classified by biochemical techniques. According to serotyping by co-agglutination test using rabbit monospecific serotype antisera, 40 (49.4%) of 81 bovine and 30 (32.3%) of 93 human GBS isolates were found as nontypeable (NT). The various biochemical properties of NT GBS isolates were statistically analyzed and isolates were classified according to cluster variables. Bovine and human NT isolates were classified in four and three different biotype patterns. It was concluded that these patterns will be a guide for the identification scheme of bovine and human GBS isolates and raised the question if there are new no-named isolates other than *Streptococcus agalactiae* in GBS?

Key Words *Group B Streptococci, Biotype, Nontypeable, Bovine, Human*

Serotiplendirilemeyen Grup B Streptokokların Biyotiplendirilmesi

ÖZET

Bu araştırmada serotiplendirilemeyen Grup B Streptokok (GBS)'ların biyokimyasal yöntemlerle sınıflandırılması amaçlandı. Sığırlardan izole edilen 81 izolatın 40 (%49.4)'ı ve 93 insan izolatının 30 (%32.3)'u tavşanlardan elde edilen serotip monospesifik antiserumların kullanıldığı koaglütinasyon testi ile serotiplendirilemedi. Serotiplendirilemeyen izolatlardan elde edilen bazı biyokimyasal test sonuçlarının istatistiksel dendogram analizleri yapılarak her biri sınıflandırmaya tabi tutuldu. Sığır orijinli izolatlar 4 farklı biyotipte sınıflandırılırken insan orijinli izolatlarda 3 farklı biyotip saptandı. Araştırma sonucunda elde edilen biyotip modellerinin sığır ve insan orijinli GBS izolatlarının identifikasyonunda yardımcı olabileceği ve GBS kapsamında *Streptococcus agalactiae* dışında adlandırılmayan başka izolatların da olabileceği kanaatine varıldı.

Anahtar Kelimeler *Grup B streptokok, Biyotip, Serotiplendirilemeyen, Sığır, İnsan*

INTRODUCTION

Group B streptococci (GBS) are a well-recognized etiological agent of bovine mastitis leading to economic losses through of the world. Clinicians focused attention on the organism as a major pathogen of neonatal sepsis and purulent meningitis (early onset and late onset) in newborns as well as adults (Schuctat and Wenger 1994).

The phenotypic assessment of GBS isolates by serotyping has traditionally been used in classifying GBS isolates. Serotyping, based on the capsular polysaccharide (CPS) and protein antigen of the cell wall, is important for inform about the epidemiological aspects and distribution of GBS in the world (Devriese 1991, Lachenauer et al. 1999, Hickman et al. 1999).

Capsular serotyping is the classic method for typing GBS in epidemiological studies. GBS is serologically classified into at least nine capsular polysaccharide antigen types (Ia, Ib, II, III, IV, V, VI, VII, VIII) and three protein antigen types (Ic, R and X) (Jelinkova 1977, Spellerberg 2000). In addition, the distribution of surface proteins, such as C alpha protein, C beta protein, Rib and the R proteins (R1 through R4), has been used to classify GBS isolates (Flores and Ferrieri 1989, Ferrieri and Flores 1997, Lindahl et al. 2005).

This classification devised for beta-haemolytic streptococci. It was unable to accommodate all GBS, because many alpha and non-haemolytic isolates did not possess the group specific antigen (Devriese, 1991). When the GBS isolates did not reacted with the serotype specific antisera, it defined as nontypeable (NT). In recent years, 7 to 32% of GBS isolates have been reported to be NT (Kong et al. 2008).

On the other hand, pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis typing and multilocus sequence typing (MLST) have been used to genetically; as well, biochemical properties used to phenotypically distinguish of GBS isolates (Devriese 1991, Kong et al. 2002, Ramaswamy et al. 2006).

Biotyping, refers to establishing the pattern of cellular enzymatic activity. It can be applied by conventional laboratory equipments. *Streptococcus agalactiae* is one of the well-known strain classified in the GBS. It can be simply identified by biochemical properties with CAMP and Na-hippurate positive and esculin negative characters (Devriese 1991). However, other NT isolates of GBS except *S. agalactiae*, could have differentiated by biochemical reactions.

The purpose of this study were to establish the numerical analysis of data related to various biochemical features

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and biotyping of NT isolates that were previously isolated from bovine milk and vaginal specimens from women.

MATERIALS and METHODS

Bacterial isolates: Eighty one GBS isolates previously isolated from healthy milk of lactating cows and 93 isolates from routine culture isolates of women vaginal specimens obtained from maternity clinics of two different hospitals were investigated in the present study. The cows and humans were distributed in widely different locations around Van Lake basin, eastern of Turkey. These isolates were recovered between 2003 and 2009. Reference strains of *S. agalactiae* serotype 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), X (24/60 Compton) and *Staphylococcus aureus* Cowan I (NCTC 8530) were kindly provided by Prof. Dr. Christoph LÄMMLER (from Institut für Pharmakologie und Toxikologie Fachbereich Veterinärmedizin der Justus Liebig Universität Giessen/Germany).

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. Monospecific antisera were obtained by adsorption with each cross-reactive GBS serotype culture (Jelinkova 1977, Ainsworth and Capley 1986, Mosabi et al. 1997). Serological typing was performed by co-agglutination method according to the recommendations of Christensen et al. (1973) with minor modifications.

Biochemical characterization: The isolates were submitted to additional phenotypic biochemical characterization tests like CAMP test, hydrolysis of Na-hippurate and esculin; production of acids from lactose, salicin, trehalose, raffinose and inulin (Carter 1984, Lenette et al. 1985, Winn et al. 2006); synthesis of N-acetyl β-D glucosaminidase, N-acetyl β-D galactosidase and N-acetyl β-D glucuronidase (Slifkin and Gil 1983, Gürtürk 1989).

Statistical analysis: The Statistical analyses were carried out with the Minitab (version 14 for windows) package program according to cluster variables.

RESULTS

According to serotyping, 40 (49.4%) of 81 bovine and 30 (32.3%) of 93 human GBS isolates could not be serotyped with co-agglutination test (Table 1).

Most differences between bovine and human NT isolates were found in CAMP, esculin, lactose, glucosaminidase, galactosidase and glucoronidase reactions. However Na-hippurate, salicin, trehalose, raffinose and inulin test results were found nearly similar (Table 2).

Bovine NT GBS isolates were classified as four distinct clusters by biochemical patterns (Figure 1). According to frequency, it was determined that biotype-IV was the most frequent type by 42.5% isolates. It was characterized as CAMP, Na-hippurate and inulin negative and other tests positive. Thirty-five percent of bovine isolates were found in biotype-III. It was determined as CAMP and inulin negative. The third frequent type of bovine GBS was biotype-I as 15%. These isolates were esculin, lactose, inulin, N-acetyl β-D glucosaminidase, N-acetyl β-D galactosidase and N-acetyl β-D glucuronidase negative. The last bovine GBS biotype was classified as biotype-II with CAMP, esculin, inulin, N-acetyl β-D glucosaminidase, N-acetyl β-D galactosidase and N-acetyl β-D glucuronidase negative (Table 3).

Table 1. Serotyping result of GBS isolates by co-agglutination test

Tablo 1. GBS izolatlarının ko-aglütinasyon testi ile serotiplendirme sonuçları

Source	n	Serotypable (%)	Nontypable (%)
Bovine	81	41 (50.6)	40 (49.4)
Human	93	63 (67.7)	30 (32.3)

Table 2. Positive reactions in investigated biochemical properties of bovine and human NT GBS isolates

Tablo 2. Sığır ve insan orijinli NT GBS izolatlarının incelenen biyokimyasal özelliklerinde pozitif reaksiyon sonuçları

	Positive reactions (%)	
Tests	Bovine (n:40)	Human (n:30)
CAMP	8 (20)	11 (36.6)
Na-hippurate	30 (75)	23 (76.6)
Esculin	26 (65)	2 (6.6)
Lactose	31 (77.5)	29 (96.6)
Salicin	40 (100)	30 (100)
Trehalose	37 (92.5)	30 (100)
Raffinose	40 (100)	30 (100)
Inulin	11 (27.5)	7 (23.3)
Glucosaminidase	12 (30)	24 (80)
Galactosidase	16 (40)	26 (86.6)
Glucoronidase	15 (37.5)	24 (80)

Table 3. Biotype patterns of 40 bovine NT GBS isolates according to biochemical tests

Tablo 3. Sığır orijinli 40 NT GBS izolatının biyokimyasal testlere göre şekillenen biyotip modelleri

	Biotype patterns			
Tests	Biotype-I	Biotype-II	Biotype-III	Biotype-IV
Frequency (%)	6 (15)	3 (7.5)	14 (35)	17 (42.5)
CAMP	+	v	-	-
Na-hippurate	+	v	v	v
Esculin	-	+	+	v
Lactose	-	-	+	+
Salicin	+	+	+	+
Trehalose	+	-	+	+
Raffinose	+	+	+	+
Inulin	v	-	v	v
Glucosaminidase	-	-	+	-
Galactosidase	-	-	+	-
Glucoronidase	-	-	+	-

v: variable (11-89%)

In the complete linkage analysis based on the tested biochemical properties, human NT GBS were classified as three distinct clusters (Figure 2). Biotype-II was the most frequent by 46.6% isolates. This type was determined as CAMP, esculin and inulin negative, but all other tests positive. Forty percent of human isolates were classified as biotype-III. It was characterized as CAMP, Na-hippurate, esculin and inulin negative, and the others positive. The third most frequent biotype was biotype-I as 13.3%. It was found only esculin and inulin negative; other tests positive (Table 4).

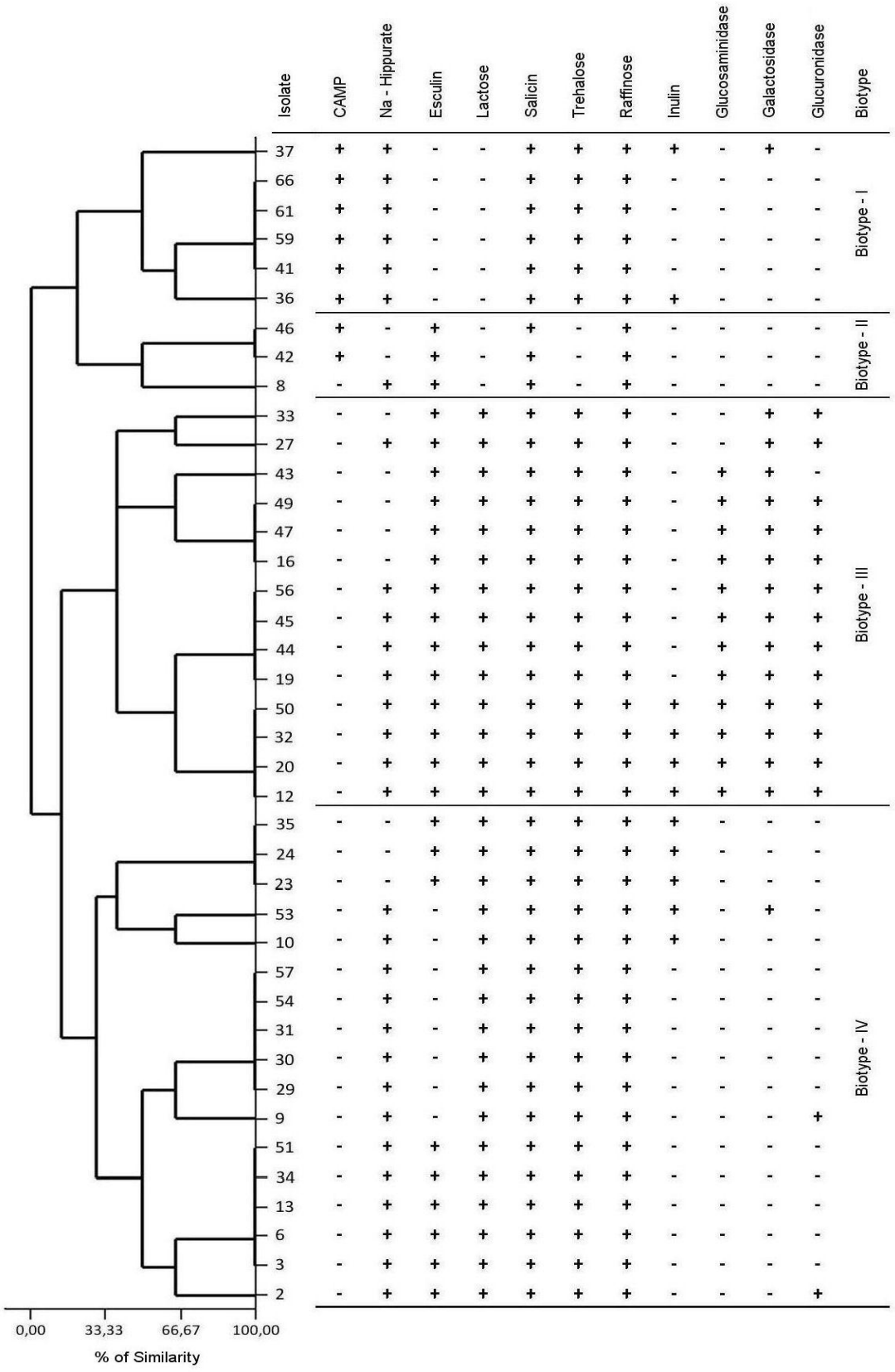

Figure 1. Biotype relationship of bovine origin NT GBS isolates estimated by clustering analysis of biochemical patterns
Şekil 1. Sığır orijinli NT GBS izolatlarının biyokimyasal modellerinin cluster analizi ve biyotip ilişkileri

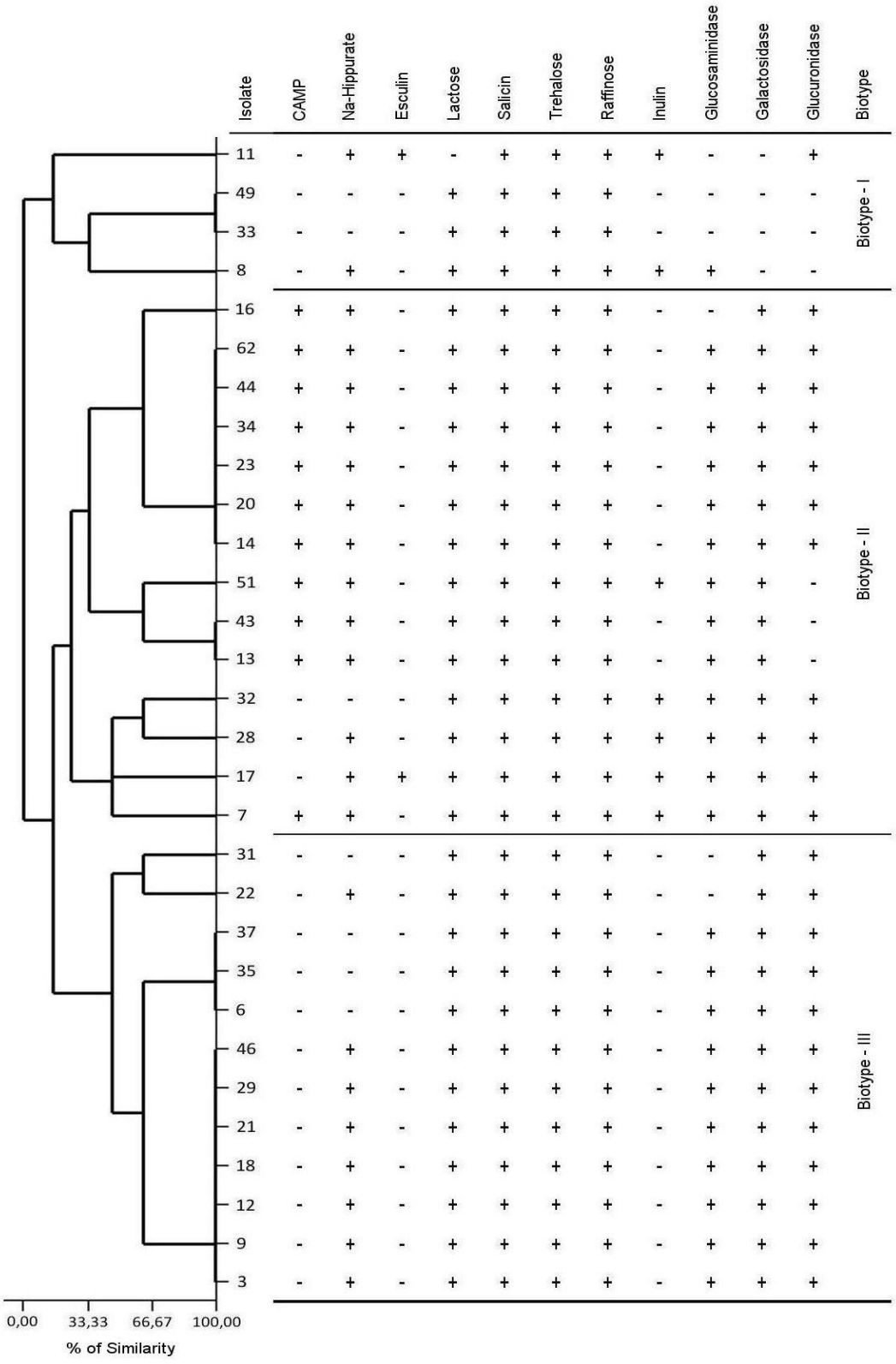

Figure 2. Biotype relationship of human origin NT GBS isolates estimated by clustering analysis of biochemical patterns
Şekil 3. İnsan orijinli NT GBS izolatlarının biyokimyasal modellerinin cluster analizi ve biyotip ilişkileri

Table 4. Biotype patterns of 30 human NT GBS isolates according to biochemical tests

Tablo 4. İnsan orijinli 30 NT GBS izolatının biyokimyasal testlere gore şekillenen biyotip modelleri

	Biotype patterns		
Tests	Biotype-I	Biotype-II	Biotype-III
Frequency (%)	4 (13.3)	14 (46.7)	12 (40)
CAMP	-	v	-
Na-hippurate	v	+	v
Esculin	v	-	-
Lactose	v	+	+
Salicin	+	+	+
Trehalose	+	+	+
Raffinose	+	+	+
Inulin	v	v	-
Glucosaminidase	v	+	v
Galactosidase	-	+	+
Glucoronidase	v	v	+

v: variable (11-89%)

DISCUSSION

Limited information was available on the epidemiology of Turkish GBS isolates recovered from bovine milk and human vaginal specimens. Previous studies on GBS isolates of human and bovine showed that the NT isolates could not be classified in routine standard diagnostic laboratories (Martinez et al 2000, Ekin and Gurturk 2006). Biological and epidemiological characteristics of the etiologic agents are essential for developing appropriate prevention programs and successful therapy (Duarte 2004).

Typeability by the polysaccharide and protein antigen of GBS isolates can be varying in different regions of the world. However, a significant proportion of GBS isolates is NT that can be differed between bovine and human origins (Slotved et al. 2003; Kong et al, 2008). It has been reported that bovine GBS isolates are found to be more nontypeable (15-55%) than human origin (1-32%) (Finch and Martin 1984; Pasaribu et al. 1985; Mosabi et al. 1997; Ekin and Gurturk 2006). In agreement with these researches, the results of this study revealed that 49.4% of the bovine isolates and 32.3% of human isolates were found NT by co-agglutination test.

S. agalactiae is the only *Streptococcus* species that has the group B antigen. Together with the unique hemolytic reactions (very small zone of lysis), two presumptive tests such as CAMP and Hippurate, are very accurate in the identification (Facklam 2002), However, many streptococcal isolates possessing group B antigen, named GBS, differs from *S. agalactiae* by biochemical characteristics such as CAMP, esculin, hippurate and haemolytic reactions. On the other hand, identification of streptococci by serological grouping suggested mostly for β- haemolytic streptococci so that α and non hemolytic strains ignored. The characteristic properties of the GBS could also be variable between β- and non β-haemolytic isolates. Thus, the identification of non β-haemolytic GBS isolates has not been clearly defined.

Devriese LA (1991) reported that lactose reactions could differentiate *Streptococcus agalactiae* human and bovine origin. In our study, almost all human isolates (96.6%) and 77.5% of bovine isolates were found to be lactose positive and human and bovine GBS isolates could not be differentiated.

It is known that the biochemical patterns of *S. agalactiae* is CAMP and Na-hippurate positive but esculin negative (Carter 1984; Facklam 2002; Winn et al. 2006). On the other hand, according to some reports, these patterns could be varying. The positive reaction of CAMP test were found between 75-100% and Na-hippurate hydrolysis as 87.5-100%, the negative reaction of esculin hydrolysis was detected as 90-100% (Müller 1967; Rund 1986; Kurl et al. 1989; Schlerka 1991; Wibawan et al. 1993; Kristin F 2002; Yildirim AÖ 2002). In this study, the positivity rates of CAMP and Na-hippurate reaction in bovine and human NT isolates were lower than those of reported. However, esculin positivity was higher in bovine isolates. These findings suggested that GBS may include other no-named strains except to *S. agalactiae*.

In our study, numerical analysis of different biochemical patterns revealed that bovine and human isolates were to classify in four and three distinct clusters indicated biotype-I, -II, -III and –IV respectively. Most of the bovine isolates were classified in biotype III and IV, which differs with their enzymatic activity. On the other hand, CAMP reaction and enzymatic activity were appeared to be characteristic for bovine GBS biotype I and biotype II respectively. Human GBS isolates were mostly classified in biotype II and III that differs by CAMP activity which appeared to be characteristic for human GBS biotype II. The patterns of each type may helpful for the identification of NT GBS isolates from bovine and human.

GBS biotype suggested in this study may be helpful for researchers and laboratories personnel for the identification of group B streptococci by routine identification techniques. Additional investigations, including sequence analysis, molecular characterization and antimicrobial susceptibility profiles of bovine and human GBS isolates in Turkey are still needed to evaluate.

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