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Novel Identification of Group B *Streptococcus* and *Enterobacter sakazakii* in Infant Suspected to Septicemia

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Abstract

Group B *Streptococcus* (GBS) is a major pathogen in neonates and pregnant women. Infant infections are significantly associated with maternal vaginal colonization with GBS during pregnancy. *Enterobacter sakazakii* is potentially a harmful bacterium, widely found in nature, transmitted from infected milk powder to infants causes various diseases. One of the biggest problems of these bacteria is the development of septicemia in newborns, causes fever, increased heart rate, respiration, and confusion. In this study, 200 blood samples were collected from infant suspected to septicemia. The bacterial genome content was then extracted by Fermehtas kits. By using the Multiplex-real time PCR technique, Group B *Streptococcus* & *Enterobacter sakazakii* were identified and their levels were determined. A total of 16 neonates (8%) with *Enterobacter sakazakii* infection and 10 infants (5%) with GBS infection were observed. Revealing the negative result of blood cultures in the current study, molecular level studies also suggested by involving other parts of the infants' body.

Introduction:

Group B *Streptococcus* (GBS) was introduced as a significant pathogen in neonates and pregnant women, a major contributor to neonatal infections (Palmeiro *et al.*, 2010; Madzivhandila *et al.*, 2011). Colonization of the genital tract occurs in 10-30% of pregnant women and is usually asymptomatic. However, it can cause urinary tract infection, septicemia, chorioamnionitis, endometritis and infectious abortion (Ulett *et al.*, 2009; Turner *et al.*, 2012). Infant's infections are significantly correlated with maternal vaginal colonization with GBS during pregnancy (Palmeiro *et al.*, 2010). The acquisition of bacteria by the baby leads to colonization of the skin or mucous membranes that occurs in 15-50% of infants born to infected mothers (Madzivhandila *et al.*, 2011). Among the infected infants, the disease progresses in 1 to 3%, and the onset of infection almost starts from the first 24 hours of birth (Convert *et al.*, 2005). Infection with GBS in infants occurs in 2 forms; early-onset disease (in infants less than 7 days) and late-onset disease (in infants one week to 3 months and rarely in older babies) (Dhanoa *et al.*, 2010; Valkenburg-van den Berg *et al.*, 2006). Premature infant infections are associated with colonization during

pregnancy and the transmission of bacteria to the baby occurs through ascites into the amniotic fluid or when passing through the delivery tube (Dhanoa *et al.*, 2010). GBS can cause infections like septicemia, meningitis, cellulitis, adenitis, conjunctivitis, pneumonia, and otitis in newborns, including sepsis and meningitis, and high mortality despite antibiotic therapy (Amirmozafari *et al.*, 2006). According to McGregor (2000), all pregnant women should perform early diagnosis of bacterial vaginosis in the first weeks of pregnancy. Leitich *et al.* (2003) showed that bacterial vaginosis is a risk factor for early labor and abortion. The prevalence of bacterial vaginosis varies widely among ethnic groups and geographical locations (Campbell *et al.*, 2000; Tolosa *et al.*, 2006) so it is suggested that their relationship with preterm labor must be investigated separately for each country (Goffinet *et al.*, 2003).

A prevalence of GBS was reported in some countries belonging to different geographical regions, with 22% in the Middle East and North Africa, 19% in Asia and the Pacific, 19% in Sub-Saharan Africa, 14% in the Americas and 12% in India and Pakistan (Stoll & Schuchat, 1998). Epidemiologic studies also showed that the colonization of

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GBS among Iranian women ranged from 1.9% to 26.7% (Fatemi *et al.*, 2010; Rabiee *et al.*, 2015).

Enterobacter sakazakii is a gram negative, negative oxidase, positive catalase, anaerobic, spores free from Enterobacteriaceae family, which is widely found on the nature and intestinal tract of humans and animals (Farmer *et al.*, 1980). It is a potentially harmful bacterium that is transmitted from infected milk to infants and due to various diseases, including meningitis, brain abscesses, necrotizing enterocolitis, encephalitis, meningococcal necrosis encephalitis, bacteremia and sepsis in newborns, especially premature newborns. Babies with suppressed immune systems, as well as low birth-weight infants, have been reported by Dumen (2010). Although there is no evidence of an epidemiological association with the infectious dose of this bacterium, the approximate number is about 1,000 organisms, which is very low and is similar to the infectious dose of pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli* O157, and *Neisseria meningitidis* (Baumgartner *et al.*, 2009; Iverse *et al.*, 2003). As stated, this pathogenic bacterium is an opportunistic and potentially pathogenic, especially in infants, the most commonly reported illnesses to occur in infants below the age of 12 months, especially preterm infants, birth deficient infants and suppressed immune neonates (MRAS-6, 2004). It is because this group of individuals is more susceptible to bacterial sepsis due to gram-negative and endotoxin bacteria associated with necrotizing enterocolitis (Beck-Sague *et al.*, 1994; Stoll *et al.*, 2004). In many cases, the importance of diseases caused by this organism is ignored due to the lack of proper sampling and the lack of advanced diagnostic facilities in clinical laboratories and the persistence of infection by *Enterobacter sakazakii* by physicians. Furthermore, in our country there are no clinical laboratories in hospitals is available for automatic culture systems, and the blood culture is carried out only through the traditional culture method. Thus the chances for proper identification and isolation of this organism are greatly reduced (CDCP, 2002). One of the biggest dangers of these bacteria is the development of septicemia in newborns, which itself create problems like fever, increased heart rate, increased breathing, confusion. There are also other symptoms that are associated with certain infections such as coughing with pneumonia or urinating with pain and kidney infection.

Methodology:

For this study, blood samples were collected from 200 infants below the age group of 12 months who were suspected of septicemia, admitted in hospitals of Shiraz and Jahrom for one year. We isolated the blood samples using 3000 rpm, then, using a sterile loop, we cultivate the whole blood on MacConkey Agar medium and incubated for 24 hour in 37°C. Further, using the Fermentans kit, the genome content of the bacteria grown on the medium was

extracted following the kit protocol. As a positive control of DNA, the purified genomes of GBS and *E.sakazakii* were prepared from the reference laboratory. Then, using the real-time method, the bacterial presence was determined in the specimens.

Extraction of DNA from blood: 200 µL of whole blood was extracted by the Fermentas DNA blood mini kit within a week of sample collection. Extracted DNA was frozen at -80°C until it underwent Real time PCR.

Primer design: based on the GBS sequences in GenBank, one pairs of primers were designed with PRI-MER3.0 (<http://frodo.wi.mit.edu/primer3>), Primer Explorer (<http://primerexplorer.jp/e/>) and NCBI primer-Blast tools (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Primers were synthesized commercially at Geneary Co. Ltd. (China). The primer 16S-23S rRNA inter-genics spacer region (ISR), within the GBS genome (Table 1).

Table 1. primers: Gene bank: FJ555494

Bacteria	Primers	
	Forward	Reverse
GBS	IGS-1, GGAAACCTG CCATTTGCGTCT	IGS-2, AATCTATTCT AGATCGTGGAAT
<i>E.sakazakii</i>	ESOMP5-F: 5'- GGTGAAGGATTTA ACCGTGAACCTT-3	ESOMP5-R: 5'- GCGCCTCGTTATCA TCCAAA-3'

Real time PCR: the PCR mixture (20 µL) contained 10 µL of 2 X SYBR Green Real-time PCR master mix (Ampliquon, Denmark), 0.5 µM of each specific primer and 1 µL of template DNA. The PCR was performed in an AB real-time RCR thermal cycler with the following program; 94°C for 1 min, 40 cycles of amplification (94°C for 30 s, 55°C for 30 s and 72°C for 20 s), followed by a final 10-min extension at 72°C. The qPCR run was done in triplicate for each assay.

Results:

In our study, Novel identification was done by using multiplex-realtime PCR on 200 suspected septicemia neonates, using primers

GBS:

"IGS-1 GGAAACCTGCCATTTGCGTCT" &
"IGS-2 AATCTATTCTAGATCGTGGAAT"

Enterobacter sakazakii:

ESOMP5-F: 5'-GGTGAAGGATTTAACCGTGAACCTT-3 &
ESOMP5-R: 5'-GCGCCTCGTTATCATCCAAA-3'

Total 16 infants from 200 (8%) with *Enterobacter sakazakii* infection and 10 infants from 200 (5%) with GBS infection were observed.

Discussion:

As mentioned earlier, *Enterobacter sakazakii* and GBS are two significant types of opportunistic pathogens for neonatal infections and can also cause various diseases in infants and sometimes cause death (Farmer *et al.*, 1980;

Figure 1. Amplification of GBS strains. The experiment performed as triplicate

Figure-2:2. melting curve for GBS primers

Palmeiro et al., 2010; Madzivhandila et al., 2011).

This study on the prevalence of *E.sakazakii* and GBS infections was important for searching prevention possibilities towards epidemics and in reducing infant mortality. Timely diagnosis and treatment of pregnant mothers to prevent fatal infections can be considered as one of the most important issues in the study of the prevalence of GBS infection.

As stated earlier, *E.sakazakii* is an infected milk formula infection that can be a contributing factor to the country's food and drug systems (Dumen, 2010). Khadijeh et al. (2013) reported on a basis of a molecular study, conducted on infants with sepsis to detect *E.sakazakii*, the infection rate was 0%, but blood culture in 1.4% of the samples was positive for *Streptococcus agalactiae* (GBS). Anthony & Okada (1977) in their study on the emergence of GBS on newborn babies in American and British hospitals reported that the incidence of this infection in neonates was between 1.3 and 3 per 1,000 live births. In a study by Behjati (1998) conducted on 114 children of below the age of 28 days, diagnosed with septicemia, found positive cultures, and some of them had multiple infections. There were 128 various bacteria and 13 of them (10.5%) were *E.sakazakii*. On the basis of a molecular study of epidemiology of beta-hemolytic GBS, colonized in the reproductive system of pregnant women, Kazem et al. (2011) reported that vaginal and rectal cultures of 21 women out of 250 (8.4%) and in the PCR assay, the colonization of GBS 24 out of 250 (9.6%) pregnant women were positive.

In our study, using Real-time PCR, we examined

simultaneously 2 important pathogens of *E.sakazakii* and GBS in susceptible septicemia neonates in Shiraz and Jahrom. Results showed that these bacteria were still potentially causing an epidemic in Fars province.

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