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ISOLATION AND RAPID DETECTION OF *CRONOBACTER SAKAZAKII* IN MILK AND MILK PRODUCTS FROM THE UNORGANIZED SECTORS OF AGRA REGION

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Abstract: *Cronobacter sakazakii* is a foodborne pathogen that commonly causes necrotizing enterocolitis, bacteremia, and meningitis, predominantly in neonates. For the present study conducted in Agra unpasteurized milk of buffalo, cow and camel and milk products such as condensed milk, processed sweets and curd were also screened for the presence of *Cronobacter sakazakii* in all the three seasons- summer, monsoon, and winter. Detection of *C. sakazakii* was done using the conventional method and 1400 isolates were obtained on specific isolation agar. Initial screening through the biochemical test and gram's staining yielded 230 presumptive isolates. Total 22 isolates, 10 from buffalo milk, 08 from condensed milk, 01 from cow milk and 03 from samples of processed sweets were isolated due to their growth pattern on different media. PCR was used to confirm the presence of virulent gene *OmpA* and housekeeping gene *GroEL*. All isolates were confirmed to be Pathogenic on sheep blood agar.

Index Terms- C. sakazakii, isolation, *OmpA*, *GroEL*.

I. INTRODUCTION

Cronobacter sakazakii is a potential food-borne opportunistic pathogen of great concern that causes life-threatening diseases and serious infections in the new-born (FAO / WHO, 2004; Bowen and Braden 2006), children and immuno-compromised individuals. It is regarded as a ubiquitous organism and has been isolated from a wide variety of foods. The natural habitat and reservoir of *Enterobacter sakazakii* remain unknown. In 2002, the International Commission on Microbiological Specifications (ICMS) for Foods had labeled *C. sakazakii* a "severe hazard for restricted populations" causing life-threatening or substantial chronic sequelae or illness of long duration. A high percentage of fatality rates have been associated with infections and diseases caused by it (Bowen and Braden, 2006). Increasing efforts have been made to certainly check the concentration of *Cronobacter* in infant foods with the recommendations of the Food and Agriculture Organization and World Health Organization (FAO /WHO, 2004). Understanding of the ecology and physiology of the organism, including its sources, transmission routes and response to environmental conditions relevant to the manufacturing process are the important microbial issues. Infant formula has been identified as one route of transmission for infection in infants. However, the primary reservoirs for subsequent contamination of foods with *Cronobacter* remain undefined due to the ubiquitous nature of these organisms. Initially it was described as a pigmented organism belonging to the *cloacae* group A and later it was known as yellow-pigmented *Enterobacter cloacae*, because the characteristic properties of this bacterium resembled the *Enterobacter cloacae* differ only in the yellow pigmentation on the TSA plate. Farmer *et al.* (1980) reported a

new species, *Enterobacter sakazakii* based on differences between *E. cloacae* and *E. sakazakii* in DNA -DNA hybridization, biochemical reactions, pigment production, and antibiotic susceptibility. DNA hybridization exhibited *E. sakazakii* to be about 50% related to *E. cloacae*. Attention of researchers are drawn because of the severe cases of infection and outbreaks that have been reported. To be caused by *E. sakazakii* are: - Meningitis (Kleiman *et al*., 1981; Himelright *et al*., 2001), Necrotizing *Enterocolitis*, Biliary sepsis, Pneumonia (Van Acker *et al*., 2001; Lai 2001), Sepsis (Muytjens *et al*., 1983), Bacteraemia (Muytjens *et al*., 1983) Brain Abscess, Malignancies (Drudy *et al*., 2006), Cerebral Infarction, Liquefaction of White Cerebral Matter, Cyst Formation, Cerebrospinal Fluid (CSF) Infection (Gurtler, 2005) The mortality rate varies from 40-80% (Bowen and Braden, 2006) and in the case of *Enterobacter* associated meningitis it is 33-80%. Neonates, particularly those of low birth weight, premature, younger than 28 weeks or immune-compromised are at greater risk of the infection by these bacteria (Drudy *et al*., 2006). In most cases the neonates die, if they survive, they develop severe neurological squeal, which can include seizures, brain abscesses, hydrocephalus, and developmental delay (Asakura *et al*., 2007). This bacterium can form biofilms, that it attaches to the stainless steel and feeding tube utensils (lversen and Forsythe, 2004; Kim *et al*., 2006). This bacteria can be found in surface water, soil, mud, grain, domestic animals, rotting wood, cattle and raw cow's milk (Muytjens and Kollee, 1990), tomatoes, fermented bread (Gassem,2002) and thermal mineral water streams (Mosso, 1994). It has also been isolated from household as well as from a wide range of medical waste. Other sources include Stethoscope and inoculated blood culture (Farmer *et al., 1980)*. In addition to food and clinical samples, *Cronobacter* spp. was isolated from various insect's intestinal tracts such as the Mexican fruit fly *Anastrepha ludens* and the stable fly *Stomoxys calcitrans*. (Kuzina *et al.*, 2001). These bacteria are very frequent and common in the powdered infant formula and they can survive for at least 2 years in powdered infant milk formula **(**Osaili and Forsythe, 2009).

II. RESEARCH METHODOLOGY

2.1 Standard Bacterial Cultures

*Cronobacter sakazakii (*MTCC 2958), *Cronobacter Muytjensii (*ATCC51329), and *Escherichia coli* (MTCC 443), were procured from "The Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology Chandigarh, INDIA".

2.2 Sample Collection

A Total of 500 samples of milk (raw buffalo milk, cow milk and camel milk) and milk products (condensed milk, processed sweets and yoghurt) were collected from the unorganized sectors of Agra city. Sampling was done seasonally: summers, winters and monsoon.

2.3 Isolation of *C. sakazakii* from raw milk and milk products

Raw milk was collected directly from udder into sterilized sample tubes; khoya, Processed Sweets and Yoghurt were collected from different local shops of Agra. Aseptic conditions were maintained during sampling. 120 Buffalo Milk, 120 Condensed Milk, 120 Processed Sweets, 90 Yoghurt, 30 raw cow milk and 20 raw camel milk were screened for the presence of *C. sakazakii*. *C. sakazakii* was isolated according to a standard protocol of FDA (2002) and Kandhai *et al.* (2004) with some modification. Briefly, 100 g/ 100ml of each sample were mixed thoroughly with 900 ml of pre-warmed sterile distilled water at 45°C, and incubated for 15-20 min in a water bath at the same temperature. Ten milliliters of each mixture were resuspended in 90 ml of *Enterobacteriaceae* enrichment broth (EE, HighMedia, India) and incubated overnight at 37°C. A loop full of the culture broth was streaked onto Violet Red Bile Glucose Agar (VRBGA, HighMedia, India) and incubated for 20- 24 h at 37°C. All colonies were streaked onto tryptic soy agar (TSA) and incubated for 24-48 h at 37°C to look for the characteristic yellow colonies of *Cronobacter* spp. All colonies that appeared yellow on TSA were picked and transferred to EE broth and again incubated for 24-48 h at 37°C. A loop full of the culture broth was streaked onto *E. sakazakii* isolation agar (ESIA, HighMedia, India) and incubated for 24-48 h at 37°C to look for the characteristic blue green colonies of *C.sakazakii.* All colonies that appeared blue green on ESIA were picked and subjected to further characterization, biochemical, pathogenicity, PCR. Confirmed cultures were preserved in EE broth containing 20% glycerol and stored at -80°C for further studies.

2.4 Gram's staining and biochemical tests

Presumptive identification of 1400 isolates was done performing gram's staining and various biochemical tests (Table 1).

2.5 Pathogenecity

Haemolysis on sheep blood agar test was studied to check the pathogenicity of *C.sakazakii* isolates.

2.6 Molecular Characterizations

The biochemically identified isolates of *C. sakazakii* were subjected to molecular identification by species specific PCR for *C. sakazakii* by using specific primers**.**

6.1 Genomic DNA extraction

The cell cultures were grown in EE broth for 24-28 Hrs. From the pure culture genomic DNA was extracted by the following procedure: 1 ml (10^8 CFU/ ml) of 24 hours pure culture of standard MTCC- 2958 and the isolates (milk and milk products) were taken in separate micro centrifuge tubes(1.5 ml). The cultures were boiled at 100°C for 10 minutes in water bath and then centrifuged at 1500g for 1 min in micro centrifuge. 200µl supernatant of this aliquot was used as the DNA template for the PCR reactions. (Nair and Venkitanarayana, 2006). Concentration and absorbance of each sample was recorded at 260 nm and the purity was by their absorbance at 260nm/280 nm. 1% Agarose gel electrophoresis was also carried out with the extracted genomic DNA at 80 V for 1 h using 1X TAE buffer for further confirmation. The gels were visualized and documented.

2.6.2 Rapid detection of *C. sakazakii* through polymerase chain reaction (PCR)

Gene amplification targeting *OMPA* gene and *GROEL (HSP 60)*

Sterile DNase RNase free water (25 µl), 10X Taq buffer (1X), 25mM MgCl2 (2 mM), 10mM dNTPs mix (0.2 mM), 100 µM Primer F (1 µM), 100 µM Primer R (1 µM); for *groEL* gene primer concentration will be (2 µM), 3 U/ µl Taq DNA polymerase (1.5 U), 100 ng / µl Template DNA (25 ng). The PCR program used was as that given by Nair and Venkitanarayana**,** 2006 for OmpA gene, by Iversen *et al*., 2004b *groEL (hsp 60)* gene. The thermo cycling conditions employed for OmpA gene were as follows: 94°C for 2 min, followed by 35 cycles comprising 94°C for 15 s, 60°C for 15 s and 72°C for 30 s. A final extension of 72°C for 5 min, for *groEL* gene 94°C for 3 min, followed by 35 cycles comprising 94°C for 30 s, 53°C for 30 s and 72°C for 1 min. A final extension of 72°C for 10 min. To standardize the reaction, the PCR assay was optimized using gradient PCR. The genomic DNA samples extracted from broth were electrophoresed on 1% agarose gels and the PCR amplified products obtained with different templates were electrophoresed on 1.5% and 2% agarose gels (Sambrook *et al.*,1989). Agarose gel of 1%, 1.5% and 2% concentration was prepared by dissolving the appropriate quantities of agarose in 1X TAE buffer (pH 8.0) in a microwave oven or by keeping in boiling water bath. Ethidium bromide (0.5 g/ml) stock solution was added directly to molten agarose solution before casting the gel.

Organism and species	Primer	Target region	Primer sequence (5'-3')	Product size	Reference
C. sakazakii	ESSF	OmpA Forward	GGATTTAACCGTGAACTTTTCC	469bp	Nair and Venkitanarayana, 2006
	ESSR	OmpA Reverse	CGCCAGCGATGTTAGAAGA	469bp	Nair and Venkitanarayana2006
C. sakazakii	*GroEL* F	Forward	GGTAGAAGAAGGCGTGGTTGC	342 bp	Iversen *et al*., 2004
	GroEL R	Reverse	ATGCATTCGGTGGTGATCATCAG	342 bp	Iversen *et al*., 2004

2.7 Statistical analysis

ANOVA and student t- test were conducted using Graph Pad Software, inc, USA.

III. RESULTS

3.1. Isolation of *C. sakazakii*

This study was planned to investigate the incidence of this organism in milk and its products in Agra region. *C. sakazakii* was isolated from the four different areas of Agra (western, eastern, northern and southern region) in three different seasons'; summer, winter and monsoon.
Samples from eastern region (Belanganj, Tajganj and Jeoni Mandi) were found to have highest occurrence of *C. sakazakii* followed by western (Shahganj) then northern (Kamla Nagar) and southern(Chipitola).In summer season highest number of this bacterium was found as compared to monsoon and winters. Total 5.4% isolates were confirmed as *C. sakazakii* (2% in buffalo milk, 1.6% Khoya/ Condensed Milk and 1.2%Processed Sweets) No significant difference was detected among the sample groups and seasons ($p > 0.05$) in *C. sakazakii* contamination.
Out of 500 samples, 63 (12.6%) samples tested positive for *C. sakazakii*. A total 1400 probable colonies were picked up on the basis of their characteristics, yellow on TSA and blue green on *C sakazakii* isolation agar. 60% confirmed isolates were from samples collected in summer, 30% in monsoon and 10% in winter season. 40% of confirmed isolates of buffalo milk were from eastern part of Agra and 40 % from western part of Agra and the rest from southern and northern. 62.5% isolates were isolated from summer samples, 25% from monsoon samples and 12.5% from samples collected in winters. 37.5% of confirmed condensed milk isolates were from northern part of Agra, 25% from southern, 25% from eastern and 12.5% from western part of Agra. 66.6% confirmed isolates were from samples collected in summer season. Samples collected in winter season did not confirm the presence of *C. sakazakii*. Confirmed isolates of processed sweets were found only in eastern parts of Agra. No presence of *C. sakazakii* was seen in camel milk and yoghurt (Table 2)

3.2 Biochemical Characterization

22 isolates confirmed to be gram negative, methyl negative, VP negative, catalase positive, nitrate positive, indole negative, oxidase negative, citrate utilization positive, TSI negative, gas production positive, motility positive, DNase positive, urease negative and by performing sugar fermentation test these isolates showed the ability to ferment glucose, raffinose, maltose (Table 1)

3.3 Pathogenic Characterization

All the isolates were positive for hemagglutination.

3.4 Molecular Characterization

The biochemically confirmed isolates were selected for molecular characterization. These 22 isolates were finally confirmed as *C. sakazakii* using ESSF and ESSR primers for *ompA* gene amplification and GroEL F and GroEL R primers for *GroEL* gene *C. muytjensii* (ATCC 51329), and *Escherichia coli* (MTCC 443) did not show the amplification of the *ompA* gene and *GroEL* gene with these set of primers. (Fig.1 and 2

IV. **DISCUSSION**

The present study has revealed the presence of *C. sakazakii* in raw Buffalo milk, khoya (condensed milk), processed sweets samples. In our study there is no incidence of *C. sakazakii* found in raw camel milk and yoghurt. New hygiene strategies are needed to reduce the occurrence of this pathogen. Many large dairy companies in Australia and other countries produce skimmed milk powder and dried milk protein products as ingredients for infant formulae or other markets with specification of *Cronobacter* limits. It has also been isolated from dairy based food, water, dried meat, and rice (Baumgartner *et al.*, 2009, Chap *et al.*, 2009). Many milk powder manufacturing companies had active programs for the control of Salmonella, including environmental monitoring programs, zoning and dry cleaning procedures in line with recommendations of the International Dairy Federation (IDF, 1991). Mullane *et al.* 2007 in their study found the prevalence of *Cronobacter* in environmental swabs from a powdered infant formulae factory. Others have examined powdered milk dust from scrapings, vacuum cleaners and spills and have reported prevalence in the range of 9–35% (Kandhai *et al*. 2004). There are basically two ways through which powdered infant formula gets contaminated: Intrinsic and extrinsic mode. In the former the bacteria is introduced into PIF during the manufacture process (Simmons *et al*., 1989) and in latter extrinsic contamination it may occur while using the contaminated utensils in the preparation of PIF (Noriega *et al*., 1990). In 2009, Baumgartner *et al.*, tested 875 bulks raw milk samples from three big milk processing companies of Switzerland. In their study they found that all the tested 875 samples were negative for *Cronobacter* spp. similar observation was made by Lehner *et al*., (2004). According to their finding milk cannot be a relevant source for *Cronobacter* .Contamination of milk powders originated most likely from the environment. Craven *et al.*, in 2010 concluded that *Cronobacter* is dispersed widely in milk powder factories. In their study they suggested that distribution is assisted by movement of air, milk powder and personnel. More isolation was observed in no processing (49%) than processing areas (29%). The foodborne pathogenic bacteria isolation is important for the development and understanding of detection methods, pathogenesis, and virulence.

In the studies for the characterization of virulent *C. sakazakii,* the β-haemolytic property is widely used as virulent properties of *C. sakazakii.* The β-haemolytic property is also widely used in the determination of virulent properties of *C. amnigenus* and *C. cloacae* (Keller *et al.,* 1998). Agglutination of erythrocytes has been considered as an important virulence characteristic (Hagberg *et al.,* 1981). Hemagglutinin production and its involvement in virulence by *E. coli, Salmonella* (Duguid and Old, 1980), *Klebsiella and Serratia* (Adegbola and Old, 1982) have been reported. There are reports about *C. amnigenus, E. cloacae* and *E. sakazakii* producing mannose sensitive hemagglutinins. The extracellular DNase production is also an important virulence characteristic of most of the pathogens usually involved in invasive infections. All the strains of Group A streptococci produce at least one extracellular DNase**,** and most strains make several distinct enzymes, DNase is an important virulence factor (Sumby *et al.,* 2005**).**). "The primer pair ESSF and ESSR was designed according to the nucleotide sequence of ompA and its flanking regions. Primer ESSF was designed complementary to a region between the promoter and transcription start site of *ompA* and primer ESSR was complementary to an internal region which was less conserved among species within the *Enterobacteriaceae*. Genomic DNA sequence from 17 strains of *E. sakazakii* and 51 strains of other gram-negative and -positive bacteria were amplified using these sets of primers. All of the *C. sakazakii* strains amplified the product of 469 bp, and none of the other strains tested amplified this product. The primary goal was to develop a PCR assay for rapid and specific detection of *C. sakazakii* from PIF (Nair and Venkitanarayana, 2006).

The incidence of the *C. sakazakii* in the raw milk and milk products indicates post processing contamination from wash water, equipment, soil, utensils, and infected handlers or possibly from the environment. Presence of the *C. sakazakii* in milk makes us cautious about to use especially for the most vulnerable section of society i.e. the neonates, immune compromised individuals and the young ones. In the raw milk the occurrence of the organism also causes concern about the overall sanitary conditions prevailing at various levels of production, processing, transportation, storage and consumption. The present study is likely to have a far fetching

implication in quality assurance measures for foods, particularly of the dairy origin, in India. There can be two type of *C. sakazakii* contamination (Friedmann, 2007). In primary contamination, contamination occurs through contact with environmental sources such as water, soil and vectors like small insects and small vertebrates. Unhygienic food processing and preparation leads to secondary contamination of food products. Iversen and Forsythe in 2003 reported that *Cronobacter* is not the part of the animal and normal human gut flora.

V. CONCLUSION

Cronobacter sakazakii are ubiquitous in nature, and milk and milk products appear to be one possible natural reservoir and thus special care should be taken while preparing infant foods or formulas in order to avoid cross-contamination from these sources. Finally, the *Cronobacter sakazakii* is very diverse as indicated by the variation in the confirmation results both phenotypic and genotypic. Among the methods, the *C. sakazakii* isolation agar could be used for putative identification of *Cronobacter* sakazakii followed by the *OmpA* and *GroEL* analysis.

VI. ACKNOWLEDGEMENT

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Table 1. Biochemical and Morphological Characterization of *Cronobacter sakazakii*

S No.	Biochemical tests	Results
1	Gram staining	**Negative**
2	Spore test	**Negative**
3	VP test	**Positive**
4	MR test	**Positive**
5	Indole	**Negative**
6	Nitrate	**Positive**
7	Citrate	**Positive**
8	Catalase	**Positive**
9	Oxidase	**Positive**
10	TSI	**Negative**
11	Gas Production	**Positive**
12	Hydrogen sulphide production	**Negative**
13	Sugar Test	**Negative**
	Adontinol	**Negative**
	Arabinose	**Positive**
	Glucose	**Positive**
	Maltose	**Positive**
	Raffinose	
14	DNAase test	**Positive**
15	Motility test	**Positive**
16	Urease test	**Negative**

Table 2. Occurrence of *C. sakazakii* in Raw Milk (Buffalo Milk, Cow Milk, and Camel Milk) and Milk Products (Khoya/ Condensed Milk, Processed Sweets, Yoghurt

S No.	Source	Number of samples analyzed	Number of Positive samples	Number of isolates	Number of isolates confirmed as *C. sakazakii*	Percentage of isolates confirmed as *C. sakazakii*	Detection rate of percentage samples
1	Buffalo Milk	120	30	500	10	2%	**25%**
2	Khoya/ Condensed Milk	120	18	500	8	1.6%	**15%**
3	Processed Sweets	120	12	250	3	1.2%	**10%**
4	Yoghurt	90	-	-	-	-	
5	Cow Milk	30	3	150	1	0.6%	
6	Camel Milk	20	-				
	Total	**500**	**63**	**1400**	**22**	**5.4%**	

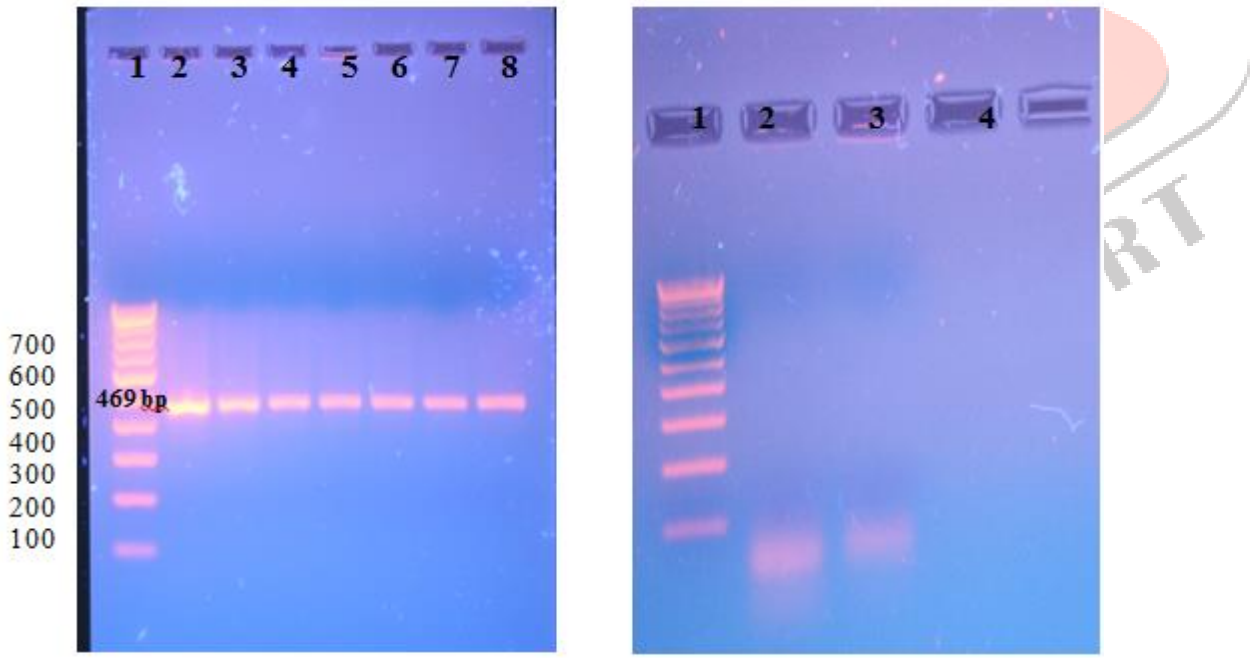
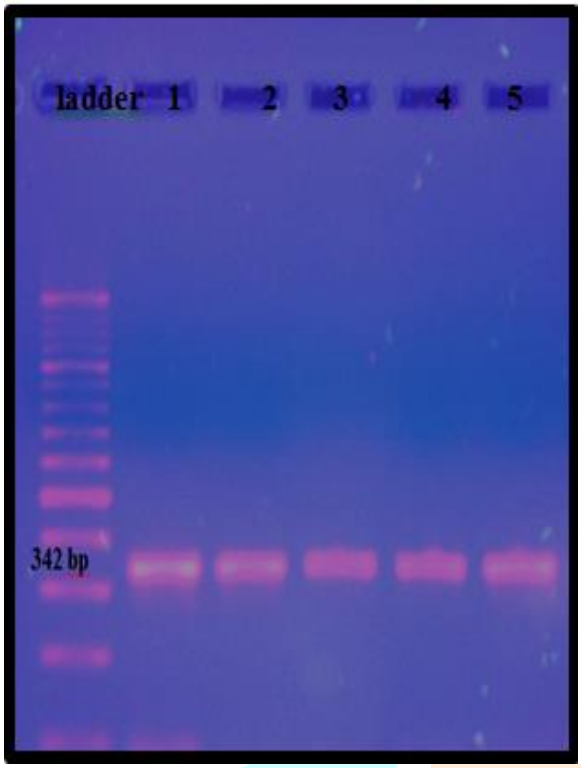


Figure 1. Detection of *C. sakazakii* by *ompA* gene unique to *C. sakazakii.* (A) Lanes 1: 100 bp ladder, 2: MTCC -2958 , Lane 3 to 8: *C. sakazakii* isolates (B) Lane 1: ladder: lane 2 *Escherichia coli* (MTCC 443) and lane 3 C. *Muytjensii* (ATCC51329) Lane 4: Blank



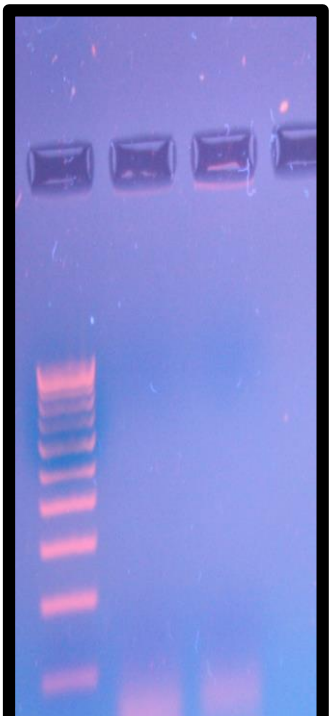


Figure 2. (A)100 bp ladder; 1: Isolate CNB1; 2: Isolate CNCM; 3: Isolate CNCN1 4: Isolate CNPS1; (B) Lane 1: ladder; lane 2: *Escherichia coli* (MTCC 443) and lane 3: C. *Muytjensii* (ATCC51329); Lane 4: Blank

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