DETECTION OF *CRONOBACTER SAKAZAKII* IN POWDERED INFANT MILK FORMULA USING REAL-TIME PCR

A RESEARCH PAPER

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF ARTS IN BIOLOGY

BY

MYOUNG-SU KIM

ADVISOR

DR. JOHN L. MCKILLIP

BALL STATE UNIVERSITY

MUNCIE, INDIANA

DECEMBER 2011

ABSTRACT

RESEARCH PAPER: Detection of *Cronobacter sakazakii* in Powdered Infant Milk Formula Using Real-time PCR

STUDENT: Myoung-Su Kim

DEGREE: Master of Arts

COLLEGE: Sciences and Humanities

DATE: December, 2011

PAGES: 15

Cronobacter sakazakii is a neonatal pathogen that has been found commonly in contaminated dried infant milk formula and milk powder. The fluorogenic selective marker, 4-Methylumbelliferyl-α-D-glucoside and secondary selective markers, sodium thiosulfate & ferric citrate have been used in differential media to indicate the presence of *C. sakazakii* based on α-D-glucosidase enzymes unique to this pathogen. This research will compare four enrichment broths for maximum recovery from powdered infant milk formula: *C. sakazakii – Enterobacter sakazakii* enrichment (ESE) broth, Tryptic Soy Broth (TSB), *Enterobacteriaceae* enrichment (EE) broth, and M-*Coliform* broth. Differential selective and nonselective agars including Trypticase Soy Agar (TSA), Violet red bile agar (VRBA), Violet red bile D-glucose agar (VRBDGA), and a newly developed KJ medium will be compared for the efficacy of isolation for the species and for optimal α-D-glucosidase activity with the fluorogenic selective marker, 4-Methylum-

belliferyl-α-D-glucoside and secondary selective markers. *C. sakazakii* strains ATCC 29544, ATCC 29004, ATCC 12868, and ATCC 51329 will be utilized as positive controls to run in artificially contaminated powder infant milk formula (PIMF) with each enrichment broth. DNA will be extracted from enrichments, which will be examined using real-time TaqMan PCR in order to compare to culture-based detection to determine relative sensitivities between the two approaches. The fluorogenic selective marker, secondary selective markers, and using a TaqMan probe PCR protocol will prove to be a rapid and specific powerful tool for the detection of *Cronobacter sakazakii* in powdered infant milk formula.

INTRODUCTION AND PROJECT GOALS

Cronobacter sakazakii, a neonatal pathogen formerly known as *Enterobacter sakazakii* has been identified epidemiologically in several illnesses such as meningitis, sepsis, and enteritis to be associated with contaminated dried infant milk formula and milk powder (Himelright, 2002; Chen, 2010). *C. sakazakii* additionally has been found in cheese, minced beef, sausage, and vegetables (Kandhai, 2006). Consumption of the pathogen is linked to a high mortality rates (33-80%) in infants (Hiroshi, 2007). Severe infections associated with high rates of meningitis have been reported in published cases (Chen, 2010). To reduce or prevent the hazards posed by *C. sakazakii,* an accurate, rapid, and highly sensitive detection protocol is needed (Drudy, 2006). Fifty-three of 57 strains (93%) of *C. sakazakii* were found to be positive for α-D-glucosidase activity (Muytjens, 1984) which was an efficient tool used to develop a differential medium (Oh, 2004). However, PCR methods are more sensitive and could permit more rapid assays than biochemical tests conducted with individual colonies (Kang, 2007). In this study, I will explore the efficiency of detection of *C. sakazakii* from powdered infant milk formula (PIMF). First, I will determine the optimal selection medium by plating *C. sakazakii* on differential medium containing 4-Methylumbelliferyl-α-D-glucoside on the differential agars from powdered infant milk formula (PIMF), inoculated with the cocktail of ATCC type strains. Second, I will investigate the specificity of a newly developed KJ medium and its usefulness for the detection of *C. sakazakii* with the distinct fluorescent colonies

on the KJ medium. I will test KJ medium that can be easily applied to detect *C. sakazakii* and will compare it with other differential media with respect to sensitivity for detection of *C. sakazakii.* Sensitivity of 4-Methylumbelliferyl-α-D-glucoside solid media and their background noise production after incubation at 37°C and the recovery and of fluorescent *C. sakazakii* colonies will be tested with total culture mixed cocktail. I will verify fluorescent and nonfluorescent colonies on KJ medium at 37°C. I will compare detection sensitivities between the culture-based selective media approach and real-time PCR on the enrichments in PIMF. Using the TaqMan probes and real-time PCR protocol for the target gene will provide a specific assay for detection of the species and for determining virulence. I will do PCR on DNA extracted from the PIMF directly, and this would allow for comparisons to be made with respect to sensitivity.

SIGNIFICANCE

A newly developed KJ medium will be tested if fluorescent colonies from *C. sakazakii* are produced. The most important thing is to determine the most sensitive detection method as mentioned above. The TaqMan real-time PCR method will prove to be a rapid, sensitive, and quantitative method for the detection of *C. sakazakii*. Does the newly developed KJ medium produce the distinct fluorescent colonies from differential enrichment broths that can be identified as *C. sakazakii*? Will ESE broth be the best selective broth for isolation of *C. sakazakii* on KJ medium? The results of this study will determine if the newly developed KJ medium may prove to be a suitable selective medium for *C. sakazakii* detection in food safety applications.

REVIEW OF THE LITERATURE

In order to reduce or prevent the hazards posed by *C. sakazakii*, the development of an accurate, rapid, and highly sensitive detection method for the identification of *C. sakazakii* in foods and environmental samples is needed. *C. sakazakii* has been detected with variety of molecular assays including PCR and fluorescent selective markers. Recently, 4-Methylumbelliferyl-α-D-glucoside was used as fluorogenic selective marker for detection of *C. sakazakii* as fluorescent colonies on some selective and nonselective media (Oh, 2004). Iversen (2007) compared enrichment broths and isolation media for the detection of *C. sakazakii.* The most accurate detection studies for PIMF and infant foods have employed DNA extraction from colonies and real-time PCR protocols with using TaqMan probes.

4-Methylumbelliferyl-α-D-glucoside**:** A substrate for α-glucosidase becomes fluorogenic by cleavage of the free 4-Methylumbelliferyl moiety when exposed to long-wave UV radiation. Among the α-glucosidase substrates, 4-nitrophenyl-α-D-glucopyranoside and 4-Methylumbelliferyl-α-D-glucoside have been tested as possible markers. The 4-nitrophenyl-α-D-glucopyranoside formed yellow-colored colonies and 4-Methylumbelliferyl-α-D-glucoside produced fluorescent colonies under UV irradiation (365 nm) (Oh, 2004). However, 4-nitrophenyl-α-D-glucopyranoside has limitations because the yellow breakdown product, 4-nitrophenol, is easily diffusible in agar, making it difficult to read (James, 1996). However, detection of α-glucosidase activity is a powerful tool to use in

developing of a differential medium.

Enterobacter sakazakii enrichment broth (ESE): All 177 known *E. sakazakii* strains grow well in ESE at 37°C (Iversen, 2007). In contrast, the growth was not detected for 3 to 13% (*n*=177) of *E. sakazakii* strains in *Enterobacteriaceae* enrichment broth (EE), modified lauryl sulfate broth (mLST), or *E. sakazakii* selective broth (ESSB) at 37°C and 44°C (Iversen, 2007). All *Enterobacteriaceae* strains grew in ESE. In the three selective enrichment broths (EE, mLST, and ESSB) the viability of four to six *E. sakazakii* strains decreased and some were unrecoverable (>6 log decline) (Carol, 2007). ESE broth was developed to facilitate comparison of the performance of *E. sakazakii* selective enrichment broths.

TaqMan Probes: The TaqMan probe or hydrolysis probe detection chemistry relies on the 5′ to 3′ exonuclease activity of DNA polymerase. This function of DNA polymerase hydrolyzes any oligonucleotide that may bind to the single-stranded portion of the DNA molecule for which the complementary strand is being synthesized. TaqMan probe chemistry exploits this property of DNA polymerase to generate a detectable signal. The hydrolysis oligonucleotide probe is labeled with both a fluorophore and a quencher molecule. In the absence of a specific target, this molecule folds up to a certain degree, which places the quencher molecule in close enough proximity to the fluorophore that the majority of any fluorophore signals emitted are immediately absorbed. When this probe binds to the complementary portion of DNA that has been generated by the previous cycles of PCR, it is hydrolyzed by DNA polymerase. Hydrolysis affords the diffusion of the fluorophore away from the quencher molecule, and generates light that is not

quenched and, therefore, is detectable. Recently, TaqMan real-time PCR assays have been developed for detection of *C. sakazakii* based on 16 rDNA sequences (Kang, 2006), and on targeting the *dnaG* and *gluA* genes (Carol, 2007), following DNA extraction (Sylviane, 2006). A real-time PCR assay targeting the *dnaG* gene, a component of the macromolecular synthesis (MMS) operon was developed by using the TaqMan probe (6-carboxyfluorescein, 6FAM–AGAGTAGTAGTTGTAGAGGCCGTGCTTCCGAAAG –TAMRA) (Seo, 2005). The fluorogenic 5' nuclease assay (TaqMan) was developed for the specific detection of *C. sakazakii* in infant formula (Seo, 2005). All "*Cronobacter*" isolates were positive using the α-D-glucosidase (*gluA*) gene, and expressed α-glucosidase activity. The α-D-glucosidase (*gluA*) gene was amplified using the following primers: EsAgf, 5'–TGA AAG CAA TCG ACA AGA AG–3'**,** and EsAgr, 5'–ACT CAT TAC CCC TCC TGA TG–3'**.** The *gluA* short fragment was amplified using primers EsAg5f (5'–TAT CAG ATC TAC CCG CGC–3') and EsAg5_5r (5'–TTG ATG CCA AGC TGT TGC–3'), resulting in a 105-bp amplicon (Carol, 2007). All *Cronobacter* isolates were 100% positive and specific using with the *dna*G and *gluA* genes PCR assays (Carol, 2007) and the study showed the results for using of 68 *Cronobacter* strains in fifty milk, soy milk, and cereal-based infant formulas. Notably for the purpose of confident identification, the *dnaG* and *gluA* genes PCR assays were 100% positive and specific for identification of *Cronobacter* strains (Carol, 2007). All *Cronobacter* isolates were positive using *dnaG* and *glu*A PCR protocols (Carol, 2007). A real-time PCR assay targeting the *dnaG* gene, a component of the macromolecular synthesis operon (MMS), was developed by using the TaqMan probe (6-carboxyfluorescein, 6FAM–AGAGTAGTAGTTGTAGA-

GGCCGTGCTTCCGAAAG–TAMRA) to increase the discriminatory power of the assay (Seo, 2005). Therefore, a set of primers (Forward: GGGATATTGTCCCCTGAAACAG, Reverse: CGACGAGAATAAGCCGCATT) and using the TaqMan probes (FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine) will be evaluated for *C. sakazakii* sensitivity (Seo, 2005).

Macromolecular synthesis (MMS) operon: The macromolecular synthesis (MMS) operon consists of three genes (*rpsU*, *dnaG*, and *rpoD*): *rpsU*, which encodes the S21 ribosomal protein, *rpsU* is replaced by *orfP23* which encodes a protein of unknown function), *dnaG*, encoding the DNA primase involved in the initiation of chromosome replication, and *rpoD*, which encodes the principal sigma subunit of RNA polymerase.

MATERIALS AND METHODS

Fluorogenic Selective Isolation: *C. sakazakii* strains ATCC 29544, ATCC 29004, ATCC 12868, and ATCC 51329 will be cultured in tryptic soy broth (TSB) overnight at 37°C before use in experiments. A portion of the overnight culture will be inoculated into each enrichment broth (100 ml) (*Enterobacteriaceae* enrichment (EE) broth, *E. sakazakii* enrichment (ESE) broth, and M-*Coliform* enrichment broth and 5 g of PIMF sample will be added to each differential enrichment broth (100 ml)). Flasks will be shaking and incubated at 37°C for 24 h. [Alternatively, each *C. sakazakii* strain will be also cultured in TSB overnight at 37°C and it will be inoculated into each enrichment broth (100 ml); this would allow for comparisons in recovery of fluorescent *C. sakazakii* colonies between each strain and cocktail strains]. All media will contain in addition the fluorogenic selective marker, 4-Methylumbelliferyl-α-glucoside (50 mg per liter) and the secondary selective markers sodium thiosulfate (1.0 g per liter) and ferric citrate (1.0 g per liter). Media used will include Violet red bile D-glucose agar (VRBDGA), Violet red bile agar (VRBA), Tryptic Soy Agar (TSA), and a newly developed KJ medium (50.0 g 4-Methylumbelliferyl-α-glucoside, 1.0 g sodium thiosulfate, 1.0 g ferric citrate, 15.0 g agar, 6.5 g disodium hydrogen phosphate, 2.0 g potassium dihydrogen phosphate, 1.5 g yeast extract, 4.0 g neutralized peptone, 12.0 g base tryptone, 4.0 g, sodium chloride, 100.0 g sucrose, and 0.5 g sodium deoxycholate dissolved in distilled water to l liter, pH 7.0 and autoclave at 12l°C for 15 min). Spreading cultures [10^{-1} to 10^{-3} dilutions] will be

used to calculate viable cells (CFU/ml) the selectivity between the colonial fluorogenic counts of *C. sakazakii* on all media. More distinct fluorescent colonies were observed under UV light with 37°C incubation rather than 30°C (Oh, 2004). Therefore, 24 h incubation at 37°C was determined as the optimal growth condition for differentiation of *C. sakazakii* (Oh, 2004).

Colony-forming units (CFU): Colonies will be obtained on all media will be counted to yield CFU/ml for comparisons as previously reported (Oh, 2004). Total bacteria colonies will be counted for comparsion between fluorescent and non-fluorescent colonies from the organism. Fluorescent *C. sakazakii* colonies and total colonies will be evaluated by mean ± standard deviations (SD) of viable counts by using SPSS.

Bacterial isolates: *C. sakazakii* strains ATCC 29544, ATCC 29004, ATCC 12868, and ATCC 51329 will be used for this study. All strains will be grown together in tryptic soy broth (TSB) at 37°C while shaking at 150 rpm. Viable *C. sakazakii* will be obtained by plating broth cultures onto nutrient agar and incubating the plates at 37°C overnight. A loopful of the enrichment broth will be streaked on VRBDGA in duplicate and incubated for 24 h at 37°C. A total of five presumptive *C. sakazakii* colonies (oxidase negative) will be subcultured on a single TSA plate and incubated for 24 h at 37°C. Yellow colonies will be selected for TSA plates, and API 20E biochemical assays will be performed to identify colonies of *C. sakazakii*.

Preparation of DNA templates for PCR: A 1 ml aliquot from the enriched EE broth samples will be centrifuged 10,000 x g for 3 min at 4°C. The supernatant will be carefully discarded and the cell pellet will be washed with 1 ml PBS (Phosphate Buffered

Saline). After centrifugation, the cell pellet will be resuspended and subjected to template extraction using the MOBIO microbial genomic DNA extraction kit (MOBIO, Solana Beach, CA). The suspension will be incubated at 65°C for 10 min and 95°C for 10 min. The samples allow to cool to room temperature. The supernatant fluids (0.5 µl) will be transferred to PCR mix (5 µl), the forward (0.5 µl) and, reverse primers (0.5 µl), TaqMan probe (0.5 µl), and water (0.5 µl). The reaction will be run at 50°C for 2 min and then 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s. The fluorescence measurements are taken in real time and are analyzed by the Rotor-Gene system.

Detection of limit of real-time PCR (standard curve generation): A 1 ml of serially diluted *C. sakazakii* strains ATCC 29544, ATCC 29004, ATCC 12868, and ATCC 51329 will be mixed with 9 ml PBS and 1.0 g of a commercial PIMF to achieve a final concentration of 10^1 to 10^8 CFU/ml reconstituted milk. The artificially inoculated the infant formula samples and pure culture samples (10^1 to 10^8 CFU/ml) will be analyzed using a real-time PCR assay as described above.

Single enrichment method: A commercial powdered milk sample (10.0 g each) will be dissolved in 90 ml of sterile water and then artificially inoculated with 100 µl of serially diluted *C. sakazakii* strains (ATCC 29544, ATCC 29004, ATCC 12868, ATCC 51329). The broth samples will be incubated for 24 h at 37°C. The single enrichment broth samples (1 ml) will be collected into 2 ml microcentrifuge tubes and used for DNA extraction.

Data analysis: The recovery of fluorescent *C. sakazakii* colonies and total colonies will be

evaluated by mean ± standard deviations (SD) of viable counts by using SPSS. Standard curves will be generated from threshold cycle numbers of a 10-fold dilution series of *C. sakazakii* in PBS (10^1 to 10^8 CFU/ml) and in powdered infant milk formula (10^1 to 10^8 CFU/ml) and correlation coefficient (R^2) by using Rotor Gene software.

REFERENCES

Chen, Pei-Chun, Zahoor, Tahir, S.W., Oh., Kang, D.H. 2010. Effect of Heat Treatment on *Cronobacter sakazakii* in Reconstituted Dried Infant Formula "Preparation Guidelines for Manufacturers." *Lett Appl Microbiol*. 49:730–737.

Drudy, D., M. O'Rourke, M. Murphy, N. Mullane, R. O'Mahony, L.Kelly.M.Fisher, S. Sanjp, P. Shannon, P. Wall, M. O'Mahony, P. Whyte, and S.Fanning. 2006. Characterizatization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *Int. J. Food Microbiol.* 110:127–134.

Hiroshi A., Morita-Ishihara, T., Yamamoto, S. and Igimi, I. 2007. Genetic characterization of thermal tolerance in *Enterobacter sakazakii. Microbial Immunol.* 51: 671–677.

Iversen, C., Angelika Lehner, Niall Mullane, John Marugg, Seamus Fanning, Roger Stephan, and Han Joosten. 2007. Identification of "Cronobacter" spp. (*Enterobacter sakazakii*). *J.Clin.Microbiol*. 45(l1):3814–3816.

Iversen, C., Mullane, N., Marugg, J., Fanning, S., Stephan, R. and Joosten, H. 2007. Identification of "*Cronobacter*" spp. (*Enterobacter sakazakii*). *J. Clin. Microbiol.* 45: 3814–3816.

Iversen, C. and Stephen J. Forsythe. 2007. Comparison of media for the isolation of *Enterobacter sakazakii*. *Appl. Environ. Microbiol*. 73(1):48–52.

James, A. L., J. D. Perry, M. Ford, L. Armstrong, and F. K. Gould. 1996. Evaluation of cyclohexen-oesculetin-β-D-galactoside and 8-hydroxyquinoline-β-D-galactoside as substrates

for the detection of β-galactosidase. *Appl. Environ. Microbiol*. 62**:**3868–3870.

Kandhai, M. C., M. W. Reij, C. Grognou, M. van Schothorst, L. G. Gorris, and M. H. Zwietering. 2006. Effects of preculturing conditions on lag time and specific growth rate of *Enterobacter sakazakii* in reconstituted powdered infant formula. *Appl. Environ. Microbiol*. 72**:**2721–2729.

Kang, Eunsil., Young Suk Nam., and Kwang Won Hong. 2007. Rapid detection of *Enterobacter sakazakii* using TaqMan real-time PCR assay. *J. Microbiol. Biotechnol*. 17:516–519.

Seo, K.H., and R.E. Brackett. 2005. Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. *J. Food Prot*. 68:59–63.

Muytjens, H. L., J. van der Ros-van de Repe, and H. A. M. van Druten. 1984. Enzymatic profiles of *Enterobacter sakazakii* and related species with special reference to the α-glucosidase reaction and reproducibility of the test system. *J. Clin. Microbiol*. 20**:**684–686.

Oh, S.W.and Kang, D.H. 2004. Fluorogenic selective and differential medium for isolation of *Enterobacter sakazakii*. *Appl. Environ. Microbiol*. 70:5692–5694.

Sylviane Derzelle and Francoise Dillasser. 2006. A robotic DNA purification protocol and real-time PCR for the detection of *Enterobacter sakazakii* in powdered infant formulate. *BMC Microbiology*. 6:100.

U.S. Food and Drug Administration. 2002. Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. [Online.] U.S. Food and Drug Administration, Gaithersburg, Md. http://www.cfsan.fda.gov/comm/mmesakaz.html.