13TH ASEAN FOOD CONFERENCE 2013

MEETING FUTURE FOOD DEMANDS: SECURITY & SUSTAINABILITY

9 - 11 SEPTEMBER 2013

MAX ATRIA, SINGAPORE EXPO

WWW.AFC2013.ORG.SG

PROGRAMME & ABSTRACTS BOOK

Organised By:

Singapore Institute of Food Science & Technology

In Conjunction With:

asean

FIESTA

Programme Book Sponsored By:

ALS

Cytotoxic Activity of Cronobacter spp. *Isolated from Indonesian Food*

Siti Nurjanah [1,2], Ratih Dewanti-Hariyadi [1,2], Sri Estuningsih [3], Maggy T. Suhartono [1]

[1]Department of Food Science and Technology, Bogor Agricultural University, IPB Darmaga Campus Bogor 16680, West Java, Indonesia, Tel/Fax: +62251 8626725
[2]SEAFAST Center , Bogor Agricultural University, IPB Darmaga Campus Bogor 16680, West Java, Indonesia, Tel/Fax: +62251 88629903
[3]Department of Veterinary Clinic Reproduction and Pathology, Bogor Agricultural University, IPB Darmaga Campus Bogor 16680, West Java, Indonesia,Tel/Fax: +62251
**Corresponding author, email:mthenawidjaja@yahoo.com*

Abstract

In previous research, twenty isolates of Cronobacter spp. were obtained from local powdered infant formula, weaning food and other dried products. Molecular showed that nineteen isolates were confirmed as Cronobacter sakazakii, while one isolate was Cronobacter muytjensii. Cronobacter virulence factors have been reported including toxin production and its cytotoxic activity. However there is limited report of its haemolytic activity. The objective of this study was to evaluate the cytotoxic activity of twenty food isolates of Cronobacter spp. obtained from Indonesia. Invitro analysis of the cytotoxic activity of Cronobacter crude toxin was conducted by MTT (Methyl Thiazol Tetrazolium) Assay which measures its inhibition to Vero cell growth. Thirteen out of twenty isolates were found to have positive cytotoxin activity which varied between 51-80% Vero cell deaths.

Key Words : Cronobacter sakazakii, Cronobacter muytjensii, cytotoxic activity, MTT Assay, Vero cell

Introduction

In Indonesia, the presence of *Cronobacter* spp. from food sources have been reported (Estuningsih et al. 2006; Meutia et al . 2008; Dewanti - Hariyadi et al. 2010; Gitapratiwi et al. 2012 and Hamdani 2012). All of the isolates were obtained from dried food products such as powdered infant formula, weaning food, starch, sugar, cocoa powder and dried spices.

Molecular identification showed that most of the isolates were confirmed as *C. sakazakii* and only one isolate confirmed as *C. muytjensii* (Gitapratiwi et al. 2012 and Hamdani 2012).

Cronobacter spp. (previously known as *Enterobacter sakazakii*) has been reported as an emerging pathogen that causes neonatal meningitis and *bacteremia* (Bowen and Braden 2006) and *necrotizing enterocolitis* (NEC) (Emami and Mittal 2012). Meningitis and NEC diseases caused by *C. sakazakii* in newborn infant have been linked to consumption of powdered infant formula (Acker et al. 2001).

Measurement of cytotoxic activity of toxin can be determined quantitatively using MTT (*Methyl Thiazol Tetrazolium*) assay method using Vero cells and qualitatively by observing the cytotoxic effect to alteration of the cells morphology. *Cronobacter* spp. have been reported to have cytotoxic activity to Vero cells as indicated by the cells damaged (Pagotto *et al.* 2003; Grecilia 2008). The objective of this study was to evaluate the cytotoxic activity of twenty food isolates of *Cronobacter* spp. obtained from Indonesia.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. *C. sakazakii* and *C. muytjensii* from SEAFAST Center (Bogor Agricultural University Indonesia) culture collection were previously isolated from dried food product. *Salmonella thypimurium* ATCC 14028 was used as a positive control for bacterial cytotoxicity.

Culture Preparation and Confirmation

All of *Cronobacter* spp. from stock culture was revived by culturing overnight at 37°C in *Brain Heart Infusion* (BHI, OXOID). The overnight culture was grown in *Druggan-*

Forsythe-Iversen (DFI, OXOID) agar medium to reconfirm their viability. *Cronobacter* spp. isolates were seen as green colonies in this medium.

Vero Cell Culture Preparation and Propagation

African green monkey kidney epithelial cells (Vero Cell Line, ATCC No. CCL-81) were used in this study. Vero cells were maintained in Eagle's minimum essential medium (Dubbeccos MEM; Invitrogen) supplemented with 2% Fetal bovine serum (FBS; Gibco), 100 U/ml fungizone and 100 µg/ml gentamycin at 37°C in 5 % CO_2 and subcultured every 3-4 days. For experiments, Vero cells suspended in DMEM supplemented with 10 % FBS were seeded in 96-well tissue culture plates and kept at 37°C and 5 % CO_2 for 24 h to form a semi confluent monolayer (5 x 10^4 cells/well).

Toxin Extraction

Overnight cultures of *Cronobacter* spp. were inoculated into 5 ml TSB (OXOID) and incubated at 37°C for 14 h. Bacterial cells were separated from the supernatant by centrifugation (3,350 x g at 4°C for 10 min). Cell-free filtrates containing the crude toxin were sterilized by filtration through 0.22-µm-pore-size membrane filters.

Cytotoxic assay

Cytotoxic activity was determined by MTT assay. An amount of 20 µl of each bacterial culture filtrates were mixed with 80 µL of DMEM. One hundred micro liters of toxin aliquots were added into semi confluent monolayer of Vero cells cultivated in 96-well tissue culture plates. The plates were incubated in 5% CO_2 atmosphere at 37°C during 48 h. After incubation, the supernatants were eliminated and monolayers were incubated with MTT salts (5 mg/ml) Sigma) as much as 10 µl per well at 37°C for 4 h. Following the incubation, the medium containing MTT was removed. Precipitated salts (formazan) produced by active

cells, were solubilized with ethanol (Merck). Aliquots of the product were transferred to each well of the new 96-well tissue culture plate. The absorbance was determined with an enzyme-linked immunosorbent assay (ELISA) reader at λ 595 nm. Experiment was performed with three well per bacterial filtrate. The cell viability was expressed as the mean of percentages of treated and untreated monolayers. Cytotoxic positive result was confirmed if there was >51% of cell death and cytotoxic negative result was confirmed if this value was less than the cytotoxic activity of the negative control.

Results and Discussion

Cytotoxic activity of the bacterial toxin is one indicator of the pathogenicity of these bacteria. The cytotoxic activity of this crude toxin was measured by MTT Assay method using Vero cells. Vero cell viability was determined by the MTT reduction that detects mitochondrial metabolic alterations as a result of the effect of cytotoxic agents on viable cell lines. The principle of this method is measuring dehydrogenase enzyme activity in mitochondria of living Vero cell by spectrophotometer. This enzyme will convert MTT reagent into formazan blue crystals. The absorbance observed at the dissolved formazan crystals indicates the number of survival cells.

MTT assay was a common method used for measuring the cytotoxic activity of either vegetative cells or toxins samples. This method was applied for determination cytotoxic activity of *Aeromonas* spp. (Krzyminska et al. 2011), *E. coli* (Ghadir et al . 2010a), *Staphylococcus* spp . and *Pseudomonas* spp . (Ghadir et al . 2010b). Application of this method for measuring the cytotoxic activity of toxin sample was reported for *Clostridium difficile* toxin (Hurtado et al . 2008), Shiga toxin (Sekino et al. 2004), *E. coli* crude toxin (Ghadir et al. 2010a), and *Aeromonas* spp. crude toxin (Coute 2007, Magda et al . 2009). Couto et al. (2007) determined positive cytotoxic results if its activity result in more than 50% Vero cell death, while Hurtado et al. (2008) confirmed positive results if the absorbance value is smaller than the absorbance value of negative control.

Table 1. Bacterial isolates used in this study

No.	Isolate Codes/ GenBank *Accession Number**	Species	Isolation Sources (from Indonesia)	Reference
1	Des c7/JF800180	*C. sakazakii*	Cornstarch	Dewanti-Hariyadi *et al.* 2010
2	Des b10/ JF800181	*C. sakazakii*	Powdered infant formulae	
3	YR c3a/JF800183	*C. sakazakii*	Weaning food	Meutia *et al.* 2008
4	YR k2a/JF800187	*C. sakazakii*	Weaning food	
5	YR t2a/JF800182	*C. sakazakii*	Powdered infant formulae	
6	YR w3/JF800185	*C. sakazakii*	Powdered infant formulae	
7	FWH b15	*C. sakazakii*	Sugar	Hamdani 2012
8	FWH d2u	*C. sakazakii*	Chili powder	
9	FWH d11	*C. muytjensii*	Caraway powder	
10	FWH b6	*C. sakazakii*	Flour	
11	FWHd16/JX535018	*C. sakazakii*	Pepper powder	
12	FWH c3	*C. sakazakii*	Tapioca	
13	FWH d1/JX535016	*C. sakazakii*	Chili powder	
14	E1	*C. sakazakii*	Weaning food	Estuningsih *et al.* 2006
15	E2	*C. sakazakii*	Weaning food	
16	E4	*C. sakazakii*	Weaning food	
17	E6	*C. sakazakii*	Weaning food	
18	E7	*C. sakazakii*	Weaning food	
19	E9	*C. sakazakii*	Weaning food	
20	E11	*C. sakazakii*	Weaning food	
21	ATCC 51329	*Enterobacter sakazakii*	References isolates	
22	ATCC 14028	*Salmonella thypimurium*	References isolates	
23	ATCC 29252	*Staphylococcus aureus*	References isolates	
24	ATCC 19433	*Enterococcus faecalis*	References isolates	

In this current study, twenty isolates of *Cronobacter sakazakii* and *Cronobacter muytjensii* showed differences in cytotoxic activity that was distributed between 32%-80% (Fig. 1). The cytotoxicity of *Salmonella typhimurium* to the human cells have been reported (Burkholder et al. 2009); therefore its activity was used as a maximum standard (100%). The growth medium (*Tryptic Soy Broth*) without toxin was used for a negative control. The positive cytotoxic results was confirmed if the cytotoxic was higher than negative control. A number of 13 out of 20 isolates (65% samples) were confirmed as positive cytotoxic isolates and 7 were grouped as negative isolates (Fig. 1) .

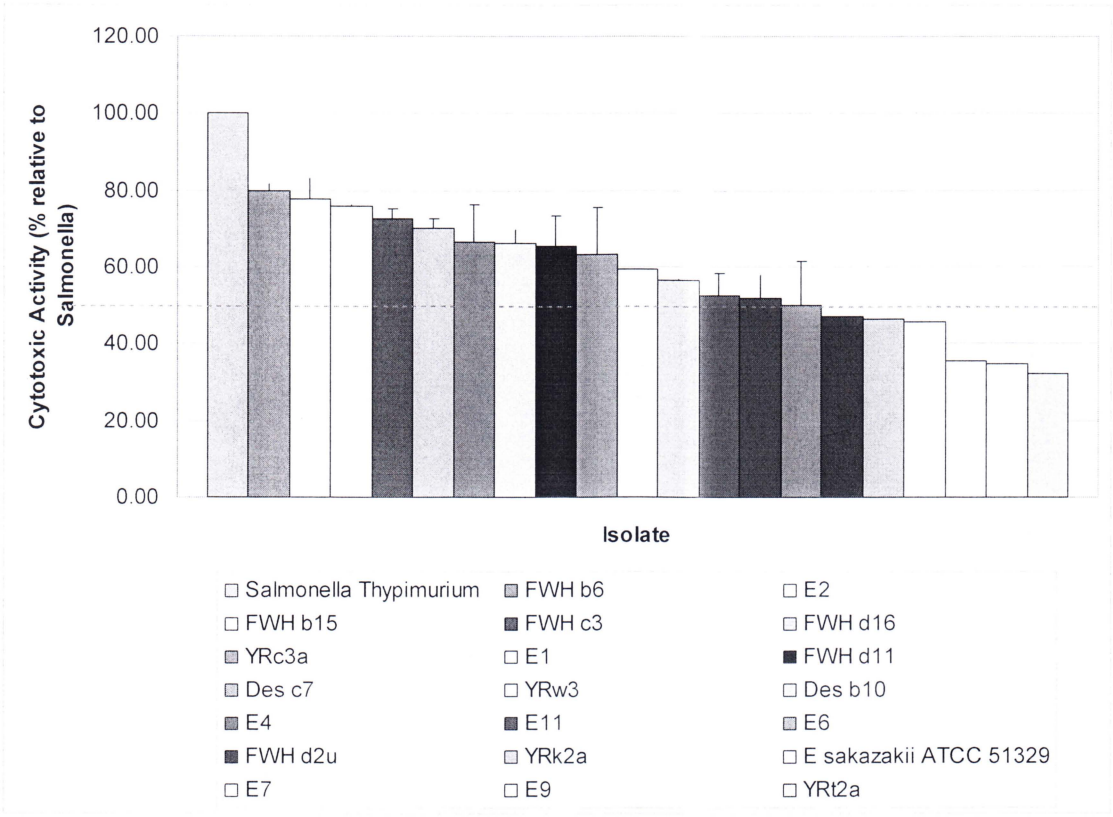

Fig. 1. Distribution of the cytotoxic activity of *Cronobacter* spp.

Five isolates with highest cytotoxicity were *C. sakazakii* FWH b6, E2, FWHb15, FWHc3 and FWHd16 (80%, 78%, 76 % , 73 % and 70 % respectively). These cytotoxicity were lower than the cytotoxicity of Shiga toxin (Sekino *et al* . 2004). Isolates with the lowest activity was YRt2a (32%) .

When compared with sources of the isolates, there was no correlation between the cytotoxicity of toxin and the food sources. When compared with the genetic diversity dendogram based on 16SrRNA gene sequences (Gitapratiwi et al. 2012), there was no clear association between positive / negative of cytotoxic activity and the genetic relationship.

Conclusion

Twenty isolates of *C. sakazakii* and *C. muytjensii* origin from food have different cytotoxic activity of their toxins. A number of 13 out of 20 isolates (65% samples) were confirmed as positive cytotoxic isolates and 7 were grouped as negative isolates Five highest

cytotoxic activity isolates were *C. sakazakii* FWH b6 , E2 , FWHb15, FWHc3 and FWHd16, while *Cronobacter muytjensii* FWHd11 belonging to moderate group.

Acknowledgements

Acknowledgements submitted to the Directorate General of Higher Education Indonesia for funding this research through Competency Grant at 2012, to SEAFAST Center and Department of Veterinary Clinic Reproduction and Pathology IPB for isolates and Vero cell collection.

References

Acker, J., Smet, F. D., Muyldermans, G., Bouhgatef, A., Naessens, A. and Lauwers, S. 2001. Outbreaks of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J Clin Microbiol. 39: 293 – 297. doi:10.1128/jcm.39.1.

Couto C.R.A,. Oliveira S.S, Queiroz M.L.P. and Freitas-Almeida AC. 2007. Interactions of clinical and environmental Aeromonas isolates with Caco-2 and HT29 intestinal epithelial cells. The Society for Applied Microbiology, Lett Appl Microbiol. 45: 405–410. doi:10.1111/j.1472-765X.2007.02199.

Bowen AB and Braden CR. 2006. Invasive Enterobacter sakazakii diseases in infants. Emerg Infect Dis. 12:1185-1189.

Dewanti-Hariyadi R, Gitapratiwi D, Meutia YR, Hidayat SH, Nurjanah S. 2010. Isolation of *Enterobacter sakazakii* (*Cronobacter* spp.) from powdered infant formula and other dried foods obtained from Bogor area. In: International Seminar on Current Issues and Challenges in Food Safety: science-based approach for food safety management. October 3-4, IPB International Conference Center, Bogor, Indonesia. Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, Bogor, Indonesia

Estuningsih S, *et al.* 2006. *Cronobactericeae* in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. J Food Protection. 69:3013-3017.

Ghadir S, Aboulwafa M M. and Hassouna NA. 2010a. Cytotoxic Activities of Some *Escherichia coli* Isolates: Possible Mechanisms and Approaches for Inhibition. J American Science. 6(10): 269-283

Ghadir S, Aboulwafa M M. and Hassouna NA. 2010b. Adherence, Invasion and Cytotoxicity of Some Bacterial Pathogens. J American Science

Gitapratiwi D, Dewanti-Hariyadi R, Hidayat SH. 2012. Genetic relatedness of *Cronobacter* spp. (*Enterobacter sakazakii*) isolated from dried food products in Indonesia. Int Food Research J. 19(4): 1745-1749.

Grecilia G. 2008. Cytotoxic testing of *Enterobacter sakazakii* isolated from weaning food on Vero cell. Skripsi. Bogor: Institut Pertanian Bogor

Hamdani FW. 2012. Evaluation of genetic diversity of local isolates *Cronobacter spp. (Enterobacter sakazakii)* from dried foods. Thesis. Bogor Agricultural University, Bogor, Indonesia.

Krzymińska S, Mokracka J, Koczura R, Ćwiertnia A,and Kaznowski A. 2011. *Aeromonas* spp.-mediated cell-contact cytotoxicity is associated with the presence of type III secretion system. Antonie Van Leeuwenhoek. 101(2): 243–251

Magda *et al.* 2009. High frequency of hemolytic and cytotoxic activity in *Aeromonas* spp. isolated from clinical, food and environmental in Rio de Janeiro, Brazil. Antonie van Leeuwenhoek (2009) 96:53–61. DOI 10.1007/s10482-009-9335-6

Meutia YR, Dewanti-Hariyadi R, Estuningsih S. 2008. Characterization of 16S rRNA gene of *Enterobacter sakazakii* isolated from powdered infant formula. In : Abstracts: Annual Seminar of Indonesian Association of Food Technologists. October 14-16, Palembang, Indonesia. Indonesian Association of Food Technologists, Jakarta, Indonesia

Pagotto FJ, Maria NW, Sabah B, and Jeffrey MF. 2003. *Enterobacter sakazakii* Infectifity and Enterotoxin Production Invitro and Invivo. J Food Protection. 66(3): 370-375.

Sekino *et al.* 2004. Characterization of a shiga-toxin 1-resistant stock of Vero cell. Microbiol Immunol. **48**(5):377-87 . PMID 15215625