Characterisation of Antibodies for the Immunochemical Detection of *Enterobacter sakazakii*

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Abstract: An indirect competitive enzyme immunoassay of *Enterobacter sakazakii* has been developed. The rabbit polyclonal antibodies to heat-labile or heat-stable antigen of the type strain *E. sakazakii* CNCTC 5739^T were prepared for these purposes. The detection limits of enzyme immunoassays were within the range 0.6–14.4 × 10^5 cells/ml. Antibodies raised to heat-labile antigen were serotype-specific. Although they contain non-specific IgG fractions binding periplasmatic and cytosol proteins, the interactions of these immunoglobulins are not manifested under conditions of ELISA developed.

Keywords: *Enterobacter sakazakii*; heat-labile antigen; IgG; ELISA; cross-reactivity

Enterobacter sakazakii is a facultative aerobic, motile, peritrichous, Gram-negative, non-sporulating, straight rod belonging to the family *Enterobacteriaceae*. Recent phylogenetic studies led to the proposal to a reclassification of *E. sakazakii* as a novel genus *Cronobacter* consisting of five species differentiable according to their phenotypic and genotypic features (Iversen *et al*. 2007, 2008). *E sakazakii* was recognised as an opportunistic pathogen and as an etiological agent in life-threatening diseases mostly in neonatal and premature infants. The most frequent mode of an infection is meningitis often complicated by ventricle compartmentalisation due to necrosis of brain tissue, brain abscess, hemorrhagic and non-hemorrhagic infarction, cyst formation and late development of hydrocephalus (Gallagher & Ball 1991; Burdette & Santos 2000; Bar-Oz *et al*. 2001). Necrotising enterocolitis and sepsis are other modes of *E. sakazakii* infection in neonatal (Arseni *et al*. 1984; Burdette & Santos 2000; Block *et al*. 2002).

The most important risk factor for human health is the incidence of *E. sakazakii* in powdered infant formula. According to the Directive of Commission (EU) No. 1441/2007 replacing the Directive of Commission (EU) No. 2073/2005 about microbiological criteria for food the absence of *E. sakazakii* is recently required in dried infant milk formulae and in dried dietary foods for special medical purposes intended for infants below six months of age. In the Czech Republic, the directive ČSN P ISO TS 22964 Milk and milk products – Detection of *Enterobacter sakazakii* is approved for isolation and identification of *E. sakazakii* in powdered milk, milk products and infant formula. The standard microbiological techniques are very sensitive, but also very laborious and time-consuming. Analysis time usually takes 6 days. These limitations emphasise the necessity for the elaboration of rapid, reliable and sensitive methods for detection of *E. sakazakii* strains in foods. Immunochemical assays and methods based on polymerase chain reaction (PCR) appear to meet such requirements. These methods do not require expensive and sophisticated instrumentation and it is possible to adapt them for field measurements.

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The principal objective of this study is to prepare high-specific polyclonal antibodies against the strains of *E. sakazakii* and to develop rapid ELISA for the detection of these pathogens in foods.

MATERIAL AND METHODS

Nutrient Broth, Plate Count Agar were purchased from Oxoid (Basingstoke, USA); Difco MIO Medium was obtained from Bencton, Dickinson & Co. (Sparks, USA); Methyl Red Voges-Proscauer Broth, Methyl Red Indicator, Decarboxylase Broth Base, Moeller, Malonate Broth, L-lysine, and dulcitol were purchased from Sigma-Aldrich (Prague, Czech Republic); Nutrient Agar, Tryptic Soy Agar, Simmons Citrate Agar, Chromocult Enterobacter Sakazakii Agar were purchased from Merck, KGaA (Darmstadt, Germany); Peptone Water with Phenol Red was obtained from HiMedia (Mumbai, India);

Table 1. List of the bacteria tested

Bacterial species	Strain number	Other strain designation	Isolated in or received from	Source
Enterobacter sakazakii	CNCTC 5739^T	ATCC 29544^T	CNCTC	child's throat
Enterobacter sakazakii	CNCTC 6830	CNCTC Eb 2/81	CNCTC	faeces
Enterobacter sakazakii	CNCTC 6831	CNCTC Eb 3/81	CNCTC	swab from bed
Enterobacter sakazakii	CNCTC 3479	H. Weiglass P-335	CNCTC	bronchial secretion
Enterobacter sakazakii	CNCTC 5740	CNCTC Eb 33/87	CNCTC	sputum
Enterobacter sakazakii	CNCTC Eb 34/87		CNCTC	faeces
Enterobacter sakazakii	CCM 3460	CDC 947-79	CCM	foot wound
Enterobacter sakazakii	CCM 3461	CDC 1565-79	CCM	abcess-base of spine
Enterobacter sakazakii	CCM 1902	ATCC 12868	CCM	*Aerobacter cloacae*
Enterobacter sakazakii	DBM 3153	148	Portugal	oregano
Enterobacter sakazakii	DBM 3154	Eb-12	Czech Republic	tofu
Enterobacter cancerogenus	CCM 4033	CNCTC 5741	CCM	skim milk (Feminar)
Enterobacter cancerogenus	CNCTC 5211	ATCC 35317	CNCTC	human arm wound
Enterobacter cloacae	CCM 1903	ATCC 10699	CCM	plasma
Enterobacter cowanii	CCM 7015^T	CIP 107300	CCM	blood, Japan
Enterobacter hormaechei	CCM 4456	ATCC 49163	CCM	blood, Hawaii
Enterobacter kobei	CCM 4078		CCM	infusion solution
Enterobacter pulveris	DBM 3158	45B	Portugal	oregano pod
Citrobacter braakii	DBM 3155	I. Hochel IHC 13	Czech Republic	short loin
Citrobacter freundii	DBM 3127	CCM 4475	DBM	frozen carrot, Czech Republic
Escherichia coli	CNCTC Eck 267/75		CNCTC	
Morganella morganii	DBM 3074		DBM	Faculty Hospital Na Bulovce
Pragia fontium	CNCTC 7010^T	ATCC 49100	CNCTC	well water
Salmonella enterica	CCM 4420	CNCTC SK 269	CCM	
Serratia marcescens	DBM 3109		DBM	Faculty Hospital Na Bulovce
Yersinia enterocolitica	CNCTC Y 14/71	J. Vandepitte, IP, 383	CNCTC	
Yersinia pseudotuberculosis	CNCTC PAPT 10/72		CNCTC	
Pseudomonas aeruginosa	DBM 3130	CCM 1959, ATCC 19429	DBM	urine
Bacillus subtilis	DBM 3006		DBM	
Micrococcus luteus	DBM 3053	CCM 169^T, ATCC 11880	DBM	fresh water, lake Sodon, Michigan
Staphylococcus aureus	DBM 3002		DBM	Faculty Hospital Na Bulovce

Bromophenol blue, *o*-phenylenediamine dihydrochloride, 3-amino-9-ethylcarbazole, formamide (99%), formaldehyde (37%), goat anti-rabbit IgG immunoglobulin-horseradish peroxidase conjugate were obtained from Sigma-Aldrich (Prague, Czech Republic).

Bacteria were obtained from the Czech Collection of Type Cultures in Prague (an acronym CNCTC), the Czech Collection of Microorganisms in Brno (an acronym CCM), and the collection of microorganisms at the Institute of Chemical Technology in Prague, Department of Biochemistry and Microbiology (an acronym DBM) or isolated from different sources (Table 1).

Phenotypic characterisation. Some phenotyping characteristics were determined by means of commercial biochemical test kit (ENTEROtest24, Pliva-Lachema, Brno, Czech Republic) according to producer's instructions. The following tests were performed using conventional manual methods. Motility was determined at 37°C after 24 h and 48 h using MIO medium. The methyl red test was done by adding 0.5 ml of indicator to the culture grown in 5 ml of Methyl Red Voges-Proscauer medium. Growth on citrate as the sole source of carbon and malonate utilisation were tested using Simmons Citrate Agar and Malonate Broth respectively, after incubation at 37°C for 24–48 hours. Lysine decarboxylase test was performed in 5 ml Decarboxylase Broth Base, Moeller containing 1% (w/v) L-lysine. The incubation took place under anaerobic conditions at 37°C for 24–48 hours. Acid production from dulcitol was tested in 5 ml of Peptone Water with Phenol Red. Dulcitol was added before the autoclaving step to the final concentration of 0.5%. Production of catalase was determined by dropping 0.05 ml of 3% hydrogen peroxide on 24 h old Nutrient Agar cultures. Yellow pigment production was observed for culture grown on Tryptic Soy Agar incubated at 25°C for 2–5 days. The α-D-glucosidase test and growth at temperature 44°C were performed using Chromocult Enterobacter Sakazakii Agar**.** The medium was inoculated with a loopful of culture and incubated at 44 ± 1°C for 24 ± 2 hours. *E. sakazakii* is α-D-glucosidase positive and forms blue-green colonies. Cytochrome oxidase test was performed with commercial strips OXItest (Pliva-Lachema, Brno, Czech Republic).

Immunogen and standard preparation. *Enterobacter sakazakii* (CNCTC 5739^T) was chosen as an immunogen for the preparation of rabbit antibodies. The microorganism was cultivated in 600 ml of Nutrient Broth (Oxoid, Basingstoke, USA) at 37°C for 24 hours. After cultivation, the cells were separated from cultivating broth by centrifugation at 5000 × g at the temperature of 4°C for 10 min and then three times washed with 20 ml of saline (0.85% NaCl). Than, pellets were resuspended in saline to the final volume of 50 ml. Exactly 25 ml of the preparation was taken and boiled for 1 hour. Heat-treated cells were centrifuged, resuspended in 25 ml of saline and stored at 0°C for 24 hours. The 0.5 ml of 37 % formaldehyde was applied into remaining 25 ml of the suspension and then stored at 0°C for 24 hours.

A similar procedure was used for the preparation of standards. After cultivation and washing, the cells were resuspended in saline to the final volume of 50 ml. The 25 ml of suspension was stored at the temperature of –80°C until used. Remaining cell suspension (25 ml) was boiled for 1 h, centrifuged, resuspended in saline and stored at –80°C. Cell concentration was determined spectrophotometrically.

Immunisation and antibody preparation. New Zealand White rabbits (male) were challenged with 10^{10} cells per dose diluted initially in Complete Freund's Adjuvant, and lately Al-Span-Oil (Sevapharma, Prague, Czech Republic) or Incomplete Freund's Adjuvant respectively. Each animal obtained six doses at three weeks intervals. Specific antibody titre dynamics was evaluated by indirect CELISA with ethanol-fixed bacteria. Titres were controlled within the whole long-time immunisation schedule.

Preparation of IgG fractions from collected sera was performed by affinity chromatography method using glass-bead immobilised protein A (Prosep A high capacity, Bioprocessing Ltd., England). After binding of sera diluted in PBS pH 7.4 and washing of a column, the globulin fraction was eluted using 0.1 mol/l citrate buffer pH 3.0. The end-use form of fractions was prepared by freeze-drying from ammonium hydrogen carbonate neutral buffer.

Indirect competitive enzyme immunoassay. The suspension of antigen (the whole cells of *E. sakazakii*) was diluted by 0.01M PBS pH 7.4 to optimal concentration (Table 2). Exact 0.1 ml of the diluted antigen was added to the wells of the polystyrene microplate, type U (Costar Corning, Cambridge, USA). The content of the wells was incubated at 37°C for 1 h followed by an additional incubation at 4°C overnight. Then 50 µl of 0.5%

Table 2. Phenotypic characteristics of *Enterobacter* sp.

Bacterial species	Strain number	Mot	Pigment	MeR	α-Glu	Orn	Ino	Ind	Dul
E. sakazakii	CNCTC 5739^T	+	+	–	+	+	+	–	–
E. sakazakii	CNCTC 5740	+	+	–	+	+	+	–	–
E. sakazakii	CNCTC Eb 34/87	+	+	–	+	–	+	–	–
E. sakazakii	CCM 3460	+	+	–	+	+	+	–	–
E. sakazakii	CCM 3479	+	+	–	+	+	+	–	–
E. sakazakii	DBM 3153	+	+	–	+	+	+	–	–
E. sakazakii	CNCTC 6830	+	+	–	+	+	–	–	–
E. sakazakii	CNCTC 6831	+	+	–	+	+	–	–	–
E. sakazakii	CCM 1902	+	+	–	+	+	+	–	–
E. sakazakii	DBM 3151	+	+	–	+	+	+	–	–
E. sakazakii	CCM 3461	+	+	–	+	+	–	+	–
E. sakazakii	DBM 3154	+	+	–	+	+	+	+	–
E. sakazakii	DBM 3152	+	+	–	+	+	+	+	–
E. pulveris	DBM 3158	+	+	+	(+)[a]	+	–	–	+

Mot – motility; Pigment – production of yellow pigment at 25°C; MeR – methyl red test; α-Glu – α-glucosidase test; Orn – ornithine decarboxylase test; Ino – acid from *myo*-inositol; Ind – indole production; Dul – acid from dulcitol

[a]positive reaction after 48 h incubation at 44°C

DBM – the collection of microorganisms at the Department of Biochemistry and Microbiology, ICT in Prague; CCM – the Czech Collection of Microorganisms, Brno; CNCTC – the Czech Collection of Type Cultures, Prague

(v/v) glutaraldehyde was added and after 15 min incubation at room temperature the reaction mixture was removed and the microplate wells were washed three times with 0.2 ml of 0.01M PBST pH 7.4. As a blocking agent 0.1 ml of 2.5% (w/v) of skimmed milk in PBS was added for 1 h at room temperature. Microplate was washed with 0.2 ml of PBST three times. In the next step 50 µl of diluted standard was added into the wells. Then 50 µl of rabbit IgG raised to heat labile antigen of *E. sakazakii* diluted with PBS to optimal concentration was added and kept to reacts for 1 h at laboratory temperature. The reaction mixture was removed and wells were washed three times with 0.2 ml of PBST. Then 0.1 ml of goat anti-rabbit IgG immunoglobulin-horseradish peroxidase conjugate diluted with 0.1% (w/v) BSA in PBST to the optimal concentration was pippeted into the microplate wells. The conjugate was kept for 1 h at the temperature of 37 °C. Finally, the wells were washed four times with 0.2 ml of PBST and then 0.1 ml of 2.7mM solution of o-phenylenediamine dihydrochloride and 0.03% (v/v) H_2O_2 in 0.05M citrate-phosphate buffer pH 5.0 was added. The enzyme reaction was terminated by addition of 50 µl 2M H_2SO_4 after 20 minutes. The absorbance was measured directly in the wells at the wavelength of 492 nm using the microplate reader SLT RainBow (Tecan, Hombrechtikon, Switzerland).

Evaluation results. The calibration curve has been calculated according to the four-parameter equation (Karpinsky 1990):

$$A = C + \frac{D - C}{1 + e^{-2(\alpha + \beta x)}}$$

where:

C, D – lower and upper asymptotes, respectively

α, β – indicators of the location and steepness of the curve

x – logarithm of analyte concentration

A – absorbance

The detection limit was calculated as the average value of absorbance at zero standard concentration minus three standard deviations.

Preparation of whole-cell lysates. A bacterial growth was harvested from single Nutrient agar plate (Oxoid, Basingstoke, USA) in 2 ml of saline

(0.85% w/v NaCl) and diluted in the same solution to the turbidity of 8 Farland's units. The volume 1.5 ml of diluted cell suspension was transferred to an Eppendorf tube and centrifuged at 8000 × g for 5 minutes. The supernatant was discarded and the remaining cell pellet was solubilized in 0.6 ml lysis buffer [10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.6 mmol Tris-HCl pH 6.8, and 0.005% (w/v) bromophenol blue]. The mixture of the lysed cells was heated at 100°C for 5 min, cooled to room temperature and then, 1.8 µl of phenylmethylsulfonylfluoride (Boehringer, Mannheim, Germany) at the concentration of 58 mg/ml was added. Finally, the sample were centrifuged at 8000 × g for 1 min before electrophoresis.

SDS-PAGE. The whole-cell lyzate preparations (10 µl per lane) were separated by polyacrylamide gel electrophoresis in discontinuous Laemmli system (Laemmli, 1970). SDS-PAGE was performed in 0.7 mm thick slab gels with the 4% stacking gel and 12% separating one. The electromigration was carried out in Mini-Protean II electrophoretic system (Bio-Rad Laboratories, Hercules, USA) under constant voltage of 200 V. After SDS-PAGE, the gels were used in an immunoblot or stained with Serva Blue G (Serva Feinbiochemica, Heidelberg, Germany). The visualisation was carried out in staining solution [0.25% (w/v) Serva Blue G, 45% (v/v) methanol, 10% (v/v) acetic acid] at room temperature for 1 hour. The destaining procedure was performed in hot deionised water. The hot water was changed three times for 30 min intervals. After destaining, the gels were stored in deionised water at 4°C.

Immunoblotting analysis of whole-cell lyzates. The electrophoretic transfer of the cell lyzates fractionated by SDS-PAGE from gel to nitrocellulose sheet (pore size 0.45 µm) was performed by means of the Trans Blot system (Bio-Rad Laboratories, Hercules, USA) using the Towbin's transfer buffer (Towbin *et al*. 1979). After the SDS-PAGE separation, the gel was immersed in the transfer buffer [25mM Tris, 192mM glycine, 20% (v/v) methanol] for 30 min and then applied to the nitrocellulose sheet. Blotting was performed under constant voltage of 100 V for 1 hour. After transfer, the nitrocellulose sheet was immersed in 5% (w/v) skimmed milk in 0.1M PBS pH 7.4 for 1 hour. The sheet was three times washed in 0.1M PBST pH 7.4 for 10 min each. Then 10 ml of rabbit IgG raised to *E. sakazakii* at the concentration of 20 µg/ml was applied and incubated at room temperature for 1 hour. The nitrocellulose sheet was washed three times in PBST and then 10 ml of swine anti-rabbit IgG immunoglobulin-horseradish peroxidase conjugate diluted with 5% (w/v) skimmed milk-PBST to the concentration of 3.8 µg/ml was applied. The icubation was performed at room temperature for 1 hour. The membrane was washed 3 times in PBST for 5 min and once in deionised water for 5 min and then immersed into substrate solution containing 0.06% (w/v) 3-amino-9-ethylcarbazole (AEC), 20% (v/v) formamide, and 0.042% (v/v) hydrogen peroxide in 0.05M citrate/phosphate buffer pH 5.0. After washing with deionised water, the membrane was dried.

RESULTS AND DISCUSSION

Thirteen from fourteen bacterial isolates obtained from various sources were identified as *Enterobacter sakazakii*. The 31 phenotypic attributes were tested. Selected results are given in Table 2. All strains were motile and positive for catalase, Voges-Proskauer, arginin dihydrolase, β-galactosidase, Simmons citrate and produced acid from cellobiose, D-glucose, D-mannose, melibiose, raffinose, L-rhamnose, sucrose, trehalose and variable for malonate utilisation. Strains hydrolysed esculin as well. Negative results were obtained for Gram staining, catalase, lysine decarboxylase, phenylalanine deaminase, urea hydrolysis, hydrogen sulfide production and for acid production from adonitol and D-sorbitol. Strain DBM 3158 originally identified as *E. sakazakii* showed methyl red test positive, a poor growth on Chromocult Enterobacter Sakazakii Agar at 44°C and an atypical α-D-glucosidase activity. According to these results and the study of 16S RNA sequence, the microorganism was re-classified as *Enterobacter pulveris* (Stephan *et al*. 2008).

Two polyclonal rabbit antibodies raised to formaldehyde treated cells of the *E. sakazakii* type strain CNCTC 5739^T (heat-labile antigen) and two polyclonal antibodies raised to the boiled cells of the same micoorganism (heat-stable antigen) have been prepared. The reciprocal antisera titres were higher than 1.0×10^5, after 119 days of the immunisation. Indirect competitive enzyme immunoassays have been developed and standardised using optimised reagent concentration obtained by checker-board titration. The reagent

Table 3. Determination of optimal reagent concentrations in ELISA of *E. sakazakii*

Immobilised Ag (10^6 cells/ml)	IgG (µg/ml)	IgG no.	IgG-Px (pg/ml)	Calibration curve				Detection limit (10^5 cells/ml)
				C	D	α	β	
3.7[a]	0.6[c]	1	75	0.171 ± 0.040	1.367 ± 0.039	4.188 ± 0.493	–0.626 ± 0.073	3.4
3.7[a]	0.3[c]	2	75	0.098 ± 0.019	1.324 ± 0.021	5.177 ± 0.361	–0.786 ± 0.054	0.6
50[b]	1.2[d]	3	75	0.278 ± 0,044	0.958 ± 0.018	7.314 ± 1.619	–0.981 ± 0.227	14.4
50[b]	1.2[d]	4	75	0.352 ± 0.053	1.069 ± 0.030	6.665 ± 1.483	–0.903 ± 0.206	8.9

Ag – antigen (whole cells of *E. sakazakii* CNCTC 5734^T); IgG – concentration of rabbit IgG in reaction mixture; IgG-Px – concentration of anti-rabbit IgG goat immunoglobulin-peroxidase conjugate
[a]untreated cells; [b]boiled cells; [c]IgG to formaldehyde treated cells; [d]IgG to boiled cells

concentrations, parameters of calibration curves and detection limits obtained are given in Table 3. After optimisation, the detection limits of ELISAs using immunoglobulins to heat labile antigens were 0.6×10^5 and 3.4×10^5 cells/ml, respectively. These values are comparable with those given in literature for the immunassays of other food-borne pathogens (Padhye & Doyle 1991; Hochel *et al*. 2007; Magliulo *et al*. 2007). Boiling of the cells reduced approximately 13 times their ability to adsorb to a polystyrene microplate surface in comparison with untreated cells (Table 3). Detection limits of assays were slightly higher if the antibodies to heat stable antigens were used. The lower values could be obtained if these antibodies will be used in sandwich enzyme immunoassay, in which IgG instead an antigen is immobilised to a solid phase. The development of the sandvich ELISA is the subject of future work. Therefore, results given below in this text are related to IgG to heat-labile antigen.

The interaction of IgG to heat-labile antigens with boiled cells of *E. sakazakii* (CNCTC 5739^T) were studied. As it follows from Figure 1, anti-

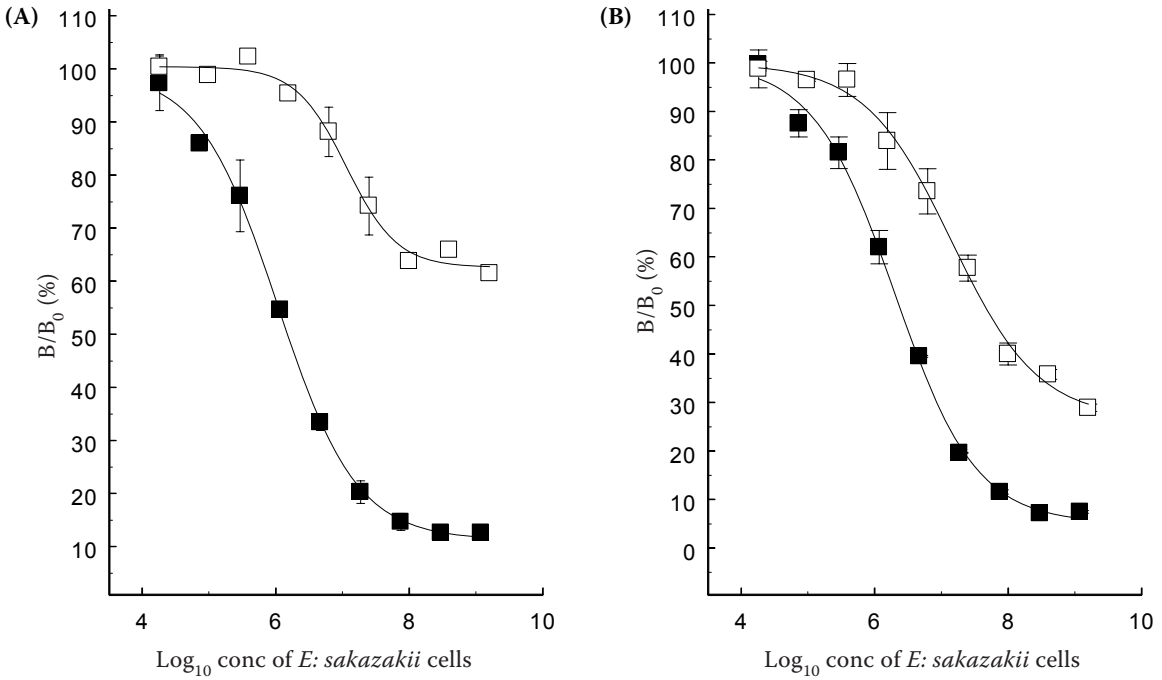

Figure 1. Interactions of rabbit IgG to formaldehyde treated cells of *E. sakazakii* (heat-labile antigen) with boiled cells (heat-stable antigen). (A) IgG No. 1; (B) IgG No 2; ■ – untreated antigen; □ – boiled antigen; B/B0 the ratio of the bound antibody in absence of analyte (B_0) and the bound antibody in the presence of given concentration of analyte. Concentration of *E. sakazakii* cells is expressed as cells/ml

Table 4. Cross-reactivity of antibodies raised to the formaldehyde treated cells of *E. sakazakii* (CNCTC 5739^T)

Bacterial species	Strain number	Cross-reactivity (%)	
		IgG No. 1	IgG No. 2
Enterobacter sakazakii	CNCTC 5739^T	+	+
Enterobacter sakazakii	CNCTC 5740	–	–
Enterobacter sakazakii	CNCTC Eb 34/87	+	+
Enterobacter sakazakii	CCM 3460	–	–
Enterobacter sakazakii	CCM 3479	–	–
Enterobacter sakazakii	DBM 3153	–	–
Enterobacter sakazakii	CNCTC 6830	–	–
Enterobacter sakazakii	CNCTC 6831	–	–
Enterobacter sakazakii	CCM 1902	–	–
Enterobacter sakazakii	DBM 3151	–	–
Enterobacter sakazakii	CCM 3461	–	–
Enterobacter sakazakii	DBM 3154	–	–
Enterobacter sakazakii	DBM 3152	–	–
Enterobacter cancerogenus	CCM 4033	–	–
Enterobacter cancerogenus	CNCTC 5211	–	–
Enterobacter cloacae	CCM 1903	–	–
Enterobacter cowanii	CCM 7015^T	–	–
Enterobacter hormaechei	CCM 4456	–	–
Enterobacter kobei	CCM 4078	–	–
Enterobacter pulveris	DBM 3158	–	–
Other bacteria		–	–

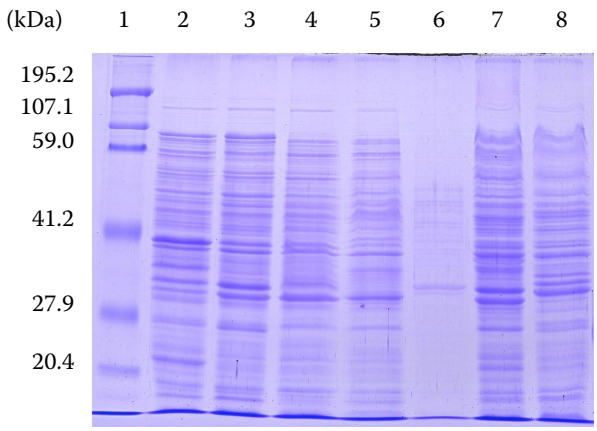

Figure 2. SDS-PAGE of whole cell lyzates. Line: 1 – SDS-PAGE molecular weight standards; 2 – *E. sakazakii* (CNCTC 5739^T); 3 – *E. sakazakii* (CNCTC Eb 34/87); 4 – *E. sakazakii* (CNCTC 6830); 5 – *E. pulveris* (DBM 3158); 6 – *E. cloacae* (CCM 1903); 7 – *E. cancerogenus* (CCM 4033); 8 – *Salmonella enterica* serotype Enteritidis (CCM 4420)

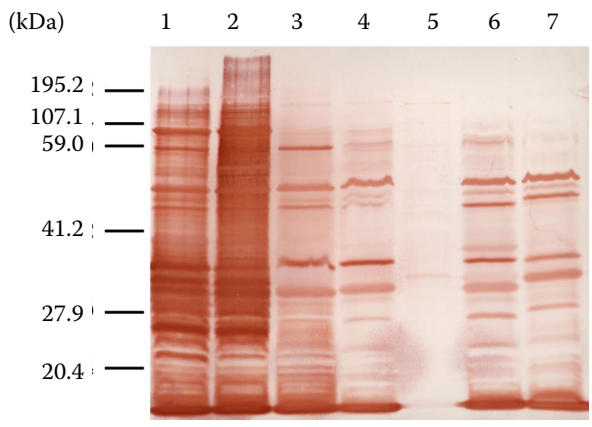

Figure 3. Interaction of rabbit IgG (no. 2) to heat-labile antigen of *E. sakazakii* with whole cell lyzate proteins of different enterobacteria. Line 1 – *E. sakazakii* (CNCTC 5739^T); 2 – *E. sakazakii* (CNCTC Eb 34/87); 3 – *E. sakazakii* (CNCTC 6830); 4 – *E. pulveris* (DBM 3158); 5 – *E. cloacae* (CCM 1903); 6 – *E. cancerogenus* (CCM 4033); 7 – *Salmonella enterica* serotype Enteritidis (CCM 4420)

bodies contain particular fractions of IgG interacting with heat-stable (somatic) antigen. Both concentration of these somatic antigen binding immunoglobulins and their average affinity differs in antibody tested. Adsorption of antibody with heated bacterial suspension of the homologous strain can remove the immunoglobulins directed against heat-stable antigen. This approach is often used to the preparation of highly specific antisera for serotyping of bacteria (Lior *et al*. 1982).

Cross-reactivity of immunoglobulins to heat-labile antigen is given in Table 4. Interactions were observed with heterogeneous strain CNCTC Eb 34/87, but not with any other bacteria. The data indicate that prepared IgGs are serotype specific. On the other hand, both antibodies bound the wide spectrum of cell proteins at molecular weight 20–195.2 kDa originating from lyzed cells of cross-reacting strain *E. sakazakii* CNCTC Eb 34/87 and other bacteria (Figures 2 and 3). Polyclonal antibodies always contain nonspecific immunoglobulins that bind various intracellular components. As it follows from our data in Table 4, however, interactions of immunoglobulins with periplasmatic and cytosol antigens are not manifested if a cell integrity is preserved under assay conditions.

Immunochemical method using single serotype-specific antibody cannot capture most strain of *E. sakazakii*, and thus it shows low specificity. This problem can be overcome using the cocktails of highly specific antibodies. It somewhat makes an optimisation procedures harder, but the undesired cross-reactivity and non-specific bindings will be suppressed during the assay. In addition to, serotype specific antibodies provide an efficient tool for *E. sakazakii* serotyping.

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