

TOOLS FOR MICROBIAL DETECTION

PERFORMANCE OF MEDIA FOR RECOVERING STRESSED CELLS OF *ENTEROBACTER SAKAZAKII* AS DETERMINED USING SPIRAL PLATING AND ECOMETRIC TECHNIQUES

(J. B. Gurtler and L. R. Beuchat)

The method used by the U.S. Food and Drug Administration to detect *Enterobacter sakazakii* in powdered infant formula requires rehydration in sterile distilled water overnight at 36°C, followed by enrichment in Enterobacteriaceae enrichment (EE) broth overnight at 36°C, surface plating and streaking on violet red bile glucose (VRBG) agar, incubating overnight at 36°C, subculturing presumptive-positive colonies in tryptic soy agar (TSA), and incubating plates for 48 - 72 h at 36°C. Yellow-pigmented presumptive positive *E. sakazakii* colonies are then subjected to confirmation tests using the API 20E biochemical identification system, which requires an additional 18 - 24 h. EE broth and VRBG agar contain selective and differential ingredients (oxgall and brilliant green in EE broth, and bile salts #3 and crystal violet in VRBG agar) that may prevent resuscitation of injured *E. sakazakii*, precluding its detection in powdered infant formula and other foods. Several media have been recently developed for detecting *E. sakazakii* in powdered infant formula. As promising as these media are for recovering *E. sakazakii* from powdered infant formula and other foods, their relative suitability for supporting resuscitation and colony development by cells after exposure to various stress environments has not been evaluated. We undertook a study with the objective to determine and compare the ability of eight agar media to resuscitate and support colony development by healthy and heat-, freeze-, acid-, alkaline-, and desiccation-stressed cells of *E. sakazakii*. Spiral plating and ecometric techniques were used to assess the performance of media.

Cells of four strains of *E. sakazakii* isolated from powder infant formula were exposed to five stress conditions: heat (55°C for 5 min), freezing (-20°C for 24 h, thawed, frozen again at -20°C for 2 h, thawed), acidic pH (3.54), alkaline pH (11.25), and desiccation in powdered infant formula (a_w 0.25, 21°C for 31 days). Control and stressed cells were spiral plated on tryptic soy agar supplemented with 0.1% pyruvate (TSAP, a non-selective control medium), Leuschner, Baird, Donald, and Cox agar (LBDC, a differential non-selective medium), Oh and Kang agar (OK), fecal coliform agar (FCA), Druggan-Forsythe-Iversen medium (DFI), violet red bile glucose (VRBG) agar, and Enterobacteriaceae enrichment (EE) agar. With the exception of desiccation-stressed cells, suspensions of stressed cells were plated on these media and on R & F *Enterobacter sakazakii* chromogenic plating medium (RF) using the ecometric technique. The order of performance of media for recovering control and heat-, freeze-, acid-, and alkaline-stressed cells by spiral plating was TSAP > LBDC > FCA > OK, VRBG > DFI > EE; the general order for recovering desiccated cells was TSAP, LBDC, FCA, OK > DFI, VRBG, EE. Using the ecometric technique, the general order of growth indices of stressed cells was TSAP, LBDC > FCA > RF, VRBG, OK > DFI, EE. Results indicated that differential, selective media vary greatly in their ability to support resuscitation and colony formation by stressed cells of *E. sakazakii*. The order of performance of media for recovering stressed cells was similar using spiral plating and ecometric techniques but results from spiral plating should be considered more conclusive.

PRODUCTION AND CHARACTERISTICS OF MONOCLONAL ANTIBODIES AGAINST SURFACE NON-FRACTION 1 ANTIGENS ON *YERSINIA PESTIS*

(T. Zhao, P. Zhao, J. Xu, D. Gottfried, and M. P. Doyle)

Yersinia pestis, the causative agent of plague, is recognized as one of the most devastating acute infectious disease experienced by humans. This notoriety is based upon the high rate of mortality, the rapid onset, and the appalling pathology associated with both the bubonic and pneumonic forms of the infection.

Rapid diagnosis is critical for the surveillance, monitoring, and control of it. Early diagnosis gives health-care providers the information necessary for effective treatment and control of disease outbreaks. Rapid identification of infectious agents has also taken on greater importance in recent years with the increased threat of biological warfare and terrorism.

Whether a large-scale outbreak of *Y. pestis* can be caused by food-related contamination has not been documented, but the potential exists. Recent studies revealed that small numbers of *Y. pestis* suspended in phosphate buffer survived 2 to 4 h after drying on stainless steel, polyethylene, or glass and beyond 48 h on paper. Cells suspended in brain heart infusion broth persisted for more than 72 h on stainless steel, polyethylene, and glass. Small numbers of cells suspended in BHI were still viable at 120 h on paper. Comprehensive studies to determine the fate of *Y. pestis* in foods need to be conducted. However, environmental studies to date indicated that *Y. pestis* can maintain viability for extended periods on four surfaces of differing characteristics that are present in health care, foodservice, or office environments.

Our initial objective was to produce monoclonal antibodies against *Y. pestis* that bind cell surface antigens other than fraction 1 which is the dominant surface antigen when the pathogen is grown at 37°C. Several different cultural conditions were evaluated to identify those that best suppress the expression of fraction 1 surface antigen and promote growth. A combination of both temperature and lower pH were used for the induction of a fraction-deficient strain.

Following immunization by fraction 1-deficient strain of *Y. pestis*, fusions were performed. Approximately 10⁷ SP2/0 cells were fused with spleen cells by 40% polyethylene. Fresh mouse red blood cells (0.5%) were used as the feeding cells in the media containing HAT supplement. The first screening included two kinds of antigens. They were *Y. pestis* (A1122) that were grown either at 37°C or 15°C. All hybridomas that recognized both *Y. pestis* grown at 37° and 15°C were further tested. The second screening included *Y. pestis* (A1122), *Y. pestis* (Harbin), and *Y. enterocolitica*. Hybridomas without cross-reaction with *Y. enterocolitica* were isotyped.

A total of ten hybridoma cell lines were isotyped. Eight cell lines produced IgG1 (heavy chain) and κ (light chain), one cell line IgG2a (heavy chain) and κ (light chain) and one cell line IgG2b (heavy chain) and κ (light chain). After isotyping, these hybridoma cell lines were expanded in plates from medium containing HAT to HT and finally without HT, and were then frozen. The supernatants collected from these cell lines were tested for cross-reaction with other species of bacteria by ELISA.

Currently, five strains of *Salmonella* Typhimurium, two of *Citrobacter freundii*, five of *Salmonella* Enteritidis, one *Escherichia coli*, five of *Salmonella* Lille, one *Salmonella* Medegridis, one *Salmonella* Montevideo, one *Salmonella* Cerro, five *Listeria monocytogenes*, four *Listeria innocua*, five *E. coli* O157:H7, five *E. coli* O111:NM, and five *E. coli* O26:H11 were tested for cross-reactivity with these MAbs. There was no significant cross-reactivity with MAbs from any of the cell lines.

More bacterial strains, especially yersiniae of species other than pestis will be tested with the MAbs by ELISA. Hybridoma cell lines that do not cross-react with bacteria other than *Y. pestis* and produce a strong titer with *Y. pestis* will be analyzed by Western blot analysis for antigen elucidation.

GREEN FLUORESCENT PROTEIN LABELING OF *LISTERIA* AND *SALMONELLA*
FOR FOOD SAFETY-RELATED STUDIES
(G. Zhang, L. Ma, and M. P. Doyle)

Many studies with foodborne bacterial pathogens require tracking of the introduced bacterial strain in order to monitor its fate in complex environments. With an easily detectable phenotype, the green fluorescent protein (GFP) gene (*gfp*), has been used to label many microorganisms for localization and gene expression studies. The objectives of this study were to label *Listeria monocytogenes*, *Listeria innocua* and *Salmonella* strains with GFP and characterize the *gfp*-labeled strains in terms of stability of label and the effect of the label on bacterial growth, two important characteristics pertinent to their intended application.

Plasmids containing the *gfp* gene were introduced into *Salmonella* and *Listeria* strains by conjugation and electroporation. Expression of GFP in labeled strains was determined by epifluorescence microscopy of colonies. Stability of the label was investigated through sequential propagations of labeled strains in the absence of antibiotic selection, and rates of plasmid-loss were calculated. Growth curves were determined comparing the parent strain with its corresponding labeled derivatives to determine the effect of *gfp*-labeling on bacterial growth. *Salmonella* strains were labeled by the calcium chloride method. *gfp*-labeling of *Listeria* by conjugation was easily performed but not universally effective for all strains, whereas electroporation was an effective method for labeling all *Listeria* strains. Plasmid stability varied among the labeled strains. When grown in non-selective media for two consecutive subcultures (ca. 40 generations), the rates of plasmid-loss among labeled *Salmonella* and *Listeria* strains ranged from 15.8%-99.9% and 8.1% -93.4%, respectively. Complete loss (>99.9%) of the plasmid was observed in some labeled strains when grown for five consecutive subcultures in the absence of selective pressure, whereas it remained stable in other cultures. Maintaining the *gfp*-plasmid had an insignificant effect on growth of most labeled strains. In conclusion, *Salmonella* and *Listeria* strains can be effectively labelled with the plasmid-borne *gfp* gene and in several isolates will be stable for many generations without adversely affecting growth rates.

MOLECULAR DETECTION AND SEROTYPING OF INFECTIOUS BRONCHITIS VIRUS FROM FTA[®] FILTER PAPER (H. Moscoso, E. O. Raybon, S. G. Thayer, and C. L. Hofacre)

FTA[®] paper is a cotton-based cellulose membrane containing lyophilized chemicals that lyses many types of bacteria and viruses. Infectious bronchitis virus (IBV) was inactivated upon contact with the FTA[®] as shown by the inability of the virus to be propagated in embryonating chicken eggs. Reverse transcriptase (RT)-polymerase chain reaction (PCR) of the SI gene showed that viral RNA in allantoic fluid remained stable after storage on FTA[®] filter cards and that the stability was time and temperature sensitive for the large (1700 base pair [bp]) but not the small (383 bp) PCR products. Analysis of the amplified products showed that molecular characterization is feasible in allantoic fluid stored on FTA[®] under nonfavorable conditions (41°C) for at least 15 days. Therefore, specimens should be first analyzed by RT-PCR with primers yielding a 1700-bp product followed by RFLP of the positive cases. Negative cases would be analyzed with primers yielding a 383-bp product (to exclude the detrimental effect of the storage conditions) followed by nucleotide sequencing of the positive cases. In conclusion, the use of FTA[®] cards for the collection, transport, and storage of IBV-containing samples is safe, inexpensive, and adequate for molecular diagnosis.

RELATIONSHIP OF MESSENGER RNA REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION SIGNAL TO *CAMPYLOBACTER* SPP. VIABILITY

(K. D. Sung, N. J. Stern, and K. L. Hiatt)

Chicken colonization by cells that have positive mRNA signal but that are noncultivable would provide a correlation in cell viability and persistence of mRNA. To examine whether this scenario exists, levels of four strains of *Campylobacter* spp., previously isolated from poultry feces, were stored for 6 to 7 wk in phosphate-buffered saline at 4°C and resulted in loss of cultivability. Cold-stored, noncultivable and heat-inactivated (60°C for 10 min) *Campylobacter* spp. produced inconsistent amplified products from RT-PCR assay, depending on the target transcripts and strains used, although all fresh cultures showed mRNA signals. For the most part, signals of mRNA species from viable but noncultivable (VBNC) and heat-killed *Campylobacter* spp. AH-1, AH-2, and CH-3 persisted. RT-PCR amplification of transcripts originating from the *tkl* and *cmp* genes and a 256-bp amplicon of haem-copper oxidase provided consistent signals, whereas transcripts from the M gene did not. Presumed VBNC and heat-inactivated *Campylobacter* spp., which produced positive mRNA signal but was not cultivable by conventional culture-based methods, did not establish colonization in the intestine of chicks 7 days after

challenge. These results lead us to question the correlation between mRNA durability with cell viability as well as the significance of the VBNC cells in environmental transmission of *Campylobacter* spp.

DNA ISOLATION FROM NONCULTIVABLE *CAMPYLOBACTER JEJUNI* ISOLATES

(K. A. Callicott, N. J. Stern, and K. L. Hiatt)

Campylobacter jejuni isolates, associated with poultry production, were subjected to 5 treatments following storage in Wang's transport medium: prolonged storage at room temperature; prolonged incubation at 42°C; multiple rounds of freezing and thawing; boiling; or contamination with *Pseudomonas aeruginosa*. Two nucleotide amplicons from the *flaA* gene (~ 400 nucleotides) and 16S rDNA (~ 800 nucleotides) were readily obtained from the noncultivable stored cells and may be used for isolate typing schemes including *flaA* short variable region (*flaA* SVR) sequencing, multilocus sequence typing (MLST) and *flaA* PCR-RFLP.

DEVELOPMENT OF RAPID DETECTION TECHNIQUE FOR PATHOGENS USING NANOROD-BASED SENSOR

(Y. W. Huang, Y. Zhao, and W. Kisaalita)

The biosecurity and safety of the food and water supply are a serious concern. Novel solutions are required for the development of rapid, reliable, and highly sensitive biosensors for the detection of low level of pathogens in food and water. Development of both the quartz crystal microbalance (QCM) and a nanoparticle-based bioassay technique for detection of *E. coli* O157:H7 and the bio-functional Au/Si nanorods for detection of Respiratory Syncytial Virus (RSV) were investigated. For *E. coli* O157:H7, the antibodies were immobilized onto gold electrodes of a QCM using a self-assembled monolayer method. Binding of *E. coli* O157:H7 cells onto the immobilized antibodies decreased the crystal resonant frequency. The difference in frequency between the phosphate buffered saline baseline and the frequency of bound *E. coli* O157:H7 demonstrated that QCM might have the potential for detecting a low level of *E. coli* O157:H7. In the nanoparticle bioassay technique, *E. coli* cells were first treated with lysozyme, then incubated with Hoechst 33258, and finally incubated with antibody-conjugated nanoparticles. A high intensity of fluorescence light produced by the nanoparticles could be observed using a fluorescence microscope. For RSV infected cells study, the Si nanorods were first fabricated by a glancing angle deposition method where the Au was sputtered onto Si nanorods. Dye was then immobilized onto the annealed Si nanorods and antibody of RSV was annealed to Au. An enhanced fluorescence signal produced by the attached dye molecules ensured a potential technique for detection of virus.