Research Note

A New Protocol for the Detection of Enterobacter sakazakii
Applied to Environmental Samples

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MS 03-58: Received 14 February 2003/Accepted 4 December 2003

ABSTRACT

Enterobacter sakazakii is a motile, peritrichous, gram-negative rod that was previously known as a yellow pigmented Enterobacter cloacae. It is documented as a rare cause of outbreaks and sporadic cases of life-threatening neonatal meningitis, necrotizing enterocolitis, and sepsis. E. sakazakii has been isolated from milk powder–based formulas, and there is thus a need to investigate whether and where E. sakazakii occurs in these manufacturing environments. For this purpose, a simple detection method was developed based on two features of E. sakazakii: its yellow pigmented colonies when grown on tryptone soy agar and its constitutive α-glucosidase, which is detected in a 4-h colorimetric assay. Using this screening method, E. sakazakii strains were isolated from three individual factories from 18 of 152 environmental samples, such as scrapings from dust, vacuum cleaner bags, and spilled product near equipment. The method is useful for routine screening of environmental samples for the presence of E. sakazakii.

Enterobacter sakazakii is a member of the family Enterobacteriaceae. Until 1980, this organism was referred to as yellow pigmented Enterobacter cloacae. It was then reclassified as a unique species based on differences from E. cloacae in DNA relatedness, the specific yellow pigment production, biochemical reactions, and antibiotic susceptibility (4). Several outbreaks or sporadic cases of either severe neonatal meningitis in premature infants or necrotizing enterocolitis have been attributed to E. sakazakii (7). In some of these, contaminated dry infant formulas have been identified as the source of E. sakazakii (3, 13). Since heat treatments such as pasteurization readily kill the organism (11), contamination must have occurred after processing. It is suspected that this microorganism is present in the environment of the processing equipment (10, 11).

Most E. sakazakii strains described in the literature were isolated from cerebrospinal fluid of the patients (7, 13). Isolates were commonly obtained by streaking onto blood or chocolate agar, using the API 20E system for identification. The isolation of E. sakazakii in milk powder is usually performed by enrichment in Enterobacteriaceae-enrichment broth, followed by plating on violet red bile glucose agar and subculturing on sheep blood agar and eosin methylene blue agar (8) or on tryptone soy agar (TSA) (12). Often, isolates were screened for the presence of DNase activity on toluidine blue agar after 2 and 7 days of incubation at 36°C and for the formation of yellow pigmented colonies on TSA at 25°C for 48 h (4, 9). An additional characteristic feature is the production of Tween 80–esterase in 3 to 8 days (1). In all, this full confirmation protocol takes more than a week. A faster and elective protocol is preferred for the detection of E. sakazakii in environmental samples in food production, in which a variety of related coliforms may be present. This study describes a simple method for the detection of E. sakazakii and its application to environmental samples collected from three milk powder production plants.

MATERIALS AND METHODS

Detection. A total of 152 dry samples were obtained from the environments of three milk powder production plants. They were collected from floor sweepings, spilled dry products, scrapings, or vacuum cleaner bags. Approximately 65% of the samples were analyzed for the presence of E. sakazakii without enrichment, whereas for 35% an enrichment step was used. In case of enrichment, 10 g of dry sample was homogenized in 90 ml of buffered peptone water (Oxoid Ltd., Basingstoke, Hampshire, UK), incubated for 18 to 20 h at 37°C, streaked onto violet red bile agar (VRBL; Oxoid), and incubated for 20 to 24 h at 37°C, as shown in Figure 1. Other samples were streaked directly onto VRBL after homogenizing. From each VRBL plate, 10 colonies typical for coliforms were purified on TSA (Oxoid). On many plates, however, fewer than 10 colonies were present, and in that case all colonies were purified on plates. The TSA plates were incubated for 48 h in daylight at room temperature. The yellow pigmentation on TSA is a characteristic feature of E. sakazakii.

Preliminary identification. Isolates were tested for oxidase, using Oxidase DrySlide OXIDASE (Becton Dickinson and Company, Sparks, Md.). Oxidase-negative isolates were further identified using the API 20E test system (bioMérieux SA, Marcy l’
For the assessment of \( \alpha \)-glucosidase activity, paranitrophenyl-D-glucopyranoside (Fluka Chemie Gmbh, Buchs, Switzerland) was dissolved in distilled water at 50 °C and added to 0.3 M (pH 7.0) phosphate buffer (Merck, KgaA, Darmstadt, Germany) in a final concentration of 4 g/liter of buffer. Individual colonies grown on TSA were suspended in 2 ml of physiological salt solution, 0.85% NaCl (Merck) in distilled water, whereupon 2 ml of the paranitrophenyl-D-glucopyranoside solution was added. Care was taken to transfer a whole colony to the test tube. The use of a standardized inoculum level of at least number 1 on the McFarland scale is recommended. The mixture was then incubated in a water bath at 37°C. The formation of the yellow colored paranitrophenyl (PNP) hydrolysate was measured after 0, 4, and 24 h using a spectrophotometer (Pharmacia Biotech, Cambridge, UK) at 405 nm. A minimal absorption of 0.3 at 405 nm after 4 h, equivalent to 16 \( \mu \)MPNP, was considered positive. Positive and negative controls were included as described below.

Ribotyping. An automated ribotyping system, the RiboPrinter (DuPont Qualicon, Wilmington, Del.), was used to confirm the identity of presumptive \( E. sakazakii \) isolates (2). In this system DNA was extracted from a colony and then digested with EcoRI into discrete-sized fragments. The DNA was then transferred to a membrane and probed with a region of the rRNA operon to reveal the pattern of rRNA genes. The pattern was recorded, digitized, and stored in a database containing 6 DuPont \( E. sakazakii \) reference fingerprints combined with the 2,000 in-house \( E. sakazakii \) fingerprints (5). Comparison of patterns in the database allows for the assessment of relatedness between \( E. sakazakii \) strains and provides further evidence of the identity of the isolates in addition to the biochemical identification.

RESULTS AND DISCUSSION

From the line environment of three milk powder plants, 152 dry samples were collected. Samples were analyzed with one of the two procedures, namely, a preenrichment step in buffered peptone water followed by streaking on VRBL agar as described in Figure 1 or a direct plating of diluted samples on VRBL agar. Coliforms were detected with both procedures. Some of these coliforms were subsequently identified as presumptive \( E. sakazakii \) on the basis of oxidase test and biochemical identification with API 20E. A total of 100 samples were not preenriched and yielded in total 30 coliforms, 7 of which were presumptive \( E. sakazakii \) strains. Fifty-two samples were subjected to preenrichment, coliforms were isolated from 33 samples, and nine of the coliforms were presumptively \( E. sakazakii \).

From the data presented here, it appears that \( E. sakazakii \) can be isolated from environmental samples with and without preenrichment. In another study, 27 samples were analyzed using both methods. Presumptive \( E. sakazakii \) strains were isolated from 13 of 27 samples without enrichment and from 16 of the same 27 samples with enrichment (data not published).

From VRBL agar, all colonies were streaked on TSA plates and were identified with API 20E. The API 20E system was not able to identify isolates obtained from 38 samples. From samples positive for \( E. sakazakii \), numerous \( E. sakazakii \) isolates were obtained in several cases that could be differentiated by their API 20E profiles and their ribotyping fingerprints. As shown in Table 1, many different species and strains of coliforms were found. Among the
Enterobacter species, the presence of α-glucosidase is known to be specific for E. sakazakii (9). The combination of the yellow colonies on TSA and positive α-glucosidase activity was therefore considered further evidence for the identity of E. sakazakii.

All 32 presumptive E. sakazakii isolates and 18 other coliforms, which had been isolated from environmental samples and had been identified using the API 20E system, were tested for their α-glucosidase activity. As shown in Table 2, 29 E. sakazakii isolates hydrolyzed paranitrophenyl-α-D-glucopyranoside within 4 h. None of the other coliforms were positive in the 4-h assay. After 24 h, the difference between E. sakazakii and the other coliforms was less clear. One Pantoea isolate and one strain of Erwinia showed substantial α-glucosidase activity to be rated positive in the 24-h assay. This indicates that the specificity of the α-glucosidase activity of E. sakazakii only applies for a short-term assay. No false-positive results were observed with the 4-h α-glucosidase assay.

Three presumptive E. sakazakii isolates were α-glucosidase negative after 4 h. These three isolates were clearly α-glucosidase negative after retesting, although they were identified as E. sakazakii by the API 20E system with a low percentage of match (80%) with the expected biochemical profile of E. sakazakii.

The presumptive E. sakazakii strains were also characterized using the ribotyping technique. Ribotyping is a method that generates a highly reproducible and precise fingerprint of bacterial rRNA genes. Using a database of reference fingerprints, it can be used to classify and identify bacteria. The technology was used here as a further tool, next to yellow pigmentation, oxidase/API 20E, and the α-glucosidase assay, to confirm the identity of E. sakazakii and also investigate relatedness among E. sakazakii isolates. The results showed that 27 presumptive E. sakazakii isolates were confirmed to be E. sakazakii by ribotyping. Two other isolates gave unclear fingerprints due mostly to the lack of DNA digestion by the EcoRI enzyme. The remaining three strains were the three α-glucosidase-negative isolates and indeed had fingerprints that were significantly different from the six reference E. sakazakii strains of the Riboprinter database. Based on the absence of α-glucosidase and the ribotyping results, it can be concluded that the three isolates identified by the API 20E system as E. sakazakii were not E. sakazakii. It should be noted that at least a 90% of biochemical reaction of the API 20E is needed for a reliable identification. The results of the ribotyping also indicated that in none of the three factories was a specific strain predominant (results not shown).

In summary, it can be concluded that the combination of yellow pigmented colonies on TSA and the presence of α-glucosidase activity detected in a 4-h assay are a good method to differentiate E. sakazakii from other coliforms. This method is useful for routine screening of environmental samples taken from milk powder factories and most likely also for their dry milk powder–based formulas (6). To ensure the detection of low numbers of E. sakazakii in environmental samples, work is currently ongoing to optimize the enrichment step.
ACKNOWLEDGMENTS

Nadine Braendlin is kindly acknowledged for her expert advice and her help in obtaining samples. The authors thank Wilma Hazeleger, Prof. dr. Leon Gorris, and Dr. M. Peterz for critically reading and reviewing the manuscript.

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