

Development and Application of a Novel Peptide Nucleic Acid Probe for the Specific Detection of *Cronobacter* Genomospecies (*Enterobacter sakazakii*) in Powdered Infant Formula^{∇†}

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Here, we report a fluorescence in situ hybridization (FISH) method for rapid detection of *Cronobacter* strains in powdered infant formula (PIF) using a novel peptide nucleic acid (PNA) probe. Laboratory tests with several *Enterobacteriaceae* species showed that the specificity and sensitivity of the method were 100%. FISH using PNA could detect as few as 1 CFU per 10 g of *Cronobacter* in PIF after an 8-h enrichment step, even in a mixed population containing bacterial contaminants.

Cronobacter strains were originally described as *Enterobacter sakazakii* (12), but they are now known to comprise a novel genus consisting of six separate genomospecies (20, 21). These opportunistic pathogens are ubiquitous in the environment and various types of food and are occasionally found in the normal human flora (11, 12, 16, 32, 47). Based on case reports, *Cronobacter* infections in adults are generally less severe than *Cronobacter* infections in newborn infants, with which a high fatality rate is associated (24).

The ability to detect *Cronobacter* and trace possible sources of infection is essential as a means of limiting the impact of these organisms on neonatal health and maintaining consumer confidence in powdered infant formula (PIF). Conventional methods, involving isolation of individual colonies followed by biochemical identification, are more time-consuming than molecular methods, and the reliability of some currently proposed culture-based methods has been questioned (28). Recently, several PCR-based techniques have been described (23, 26, 28–31, 38). These techniques are reported to be efficient even when low levels of *Cronobacter* cells are found in a sample (0.36 to 66 CFU/100 g). However, PCR requires DNA extraction and does not allow direct, in situ visualization of the bacterium in a sample.

Fluorescence in situ hybridization (FISH) is a method that is commonly used for bacterial identification and localization in samples. This method is based on specific binding of nucleic acid probes to particular DNA or RNA target regions (1, 2). rRNA has been regarded as the most suitable target for bacterial FISH, allowing differentiation of potentially viable cells. Traditionally, FISH methods are based on the use of conventional DNA oligonucleotide probes, and a commercial system,

VIT-E sakazakii (Vermicon A.G., Munich, Germany), has been developed based on this technology (25). However, a recently developed synthetic DNA analogue, peptide nucleic acid (PNA), has been shown to provide improved hybridization performance compared to DNA probes, making FISH procedures easier and more efficient (41). Taking advantage of the PNA properties, FISH using PNA has been successfully used for detection of several clinically relevant microorganisms (5, 15, 17, 27, 34–36).

***Cronobacter* probe design and theoretical evaluation.** The Primrose program (<http://www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html>) coupled with the 16S rRNA databases of Ribosomal Database Project II (version 9.55; <http://rdp.cme.msu.edu/html>) (3, 8) was employed to identify oligonucleotides that potentially could be used as probes to detect *Cronobacter*. Two oligonucleotides capable of detecting all *Cronobacter* strains with no nontarget sequence matches with Primrose were identified. The oligonucleotide that had a lower number of nontarget sequences when one nucleotide mismatch was allowed and also had a higher G+C content (and thus higher predicted melting temperature) was selected for further study. The PNA oligomer sequence obtained was 5'-TGC AGG ATT CTC TGG-3'. This probe hybridizes between positions 971 and 985 of the *Cronobacter* sp. strain ATCC 29544 16S rRNA sequence and was designated SakPNA971. The sequence was synthesized (Panagene, Daejeon, South Korea), and the oligonucleotide N terminus was attached to an Alexa Fluor 594 molecule via a double AEEA linker.

The theoretical specificity and sensitivity of the probe were evaluated further with the updated and comprehensive databases available at Ribosomal Database Project II and at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). The values were determined as previously reported by Guimarães and coworkers (15). Searches confirmed that SakPNA971 detected not only all 99 *E. sakazakii* or *Cronobacter* sp. sequences but also 11 nontarget sequences with a total of 110 matches (last accession date,

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April 2008). Therefore, the estimated theoretical specificity and sensitivity were 90 and 100%, respectively.

The 11 non-*Cronobacter* strains detected by SakPNA971 were identified as 7 *Acidithiobacillus* sp. strains (accession numbers S000342376, S000392292, S000467258, S000712736, S000712737, S000722390, and S000859142), 3 uncultured *Pseudomonadales* strains (accession numbers S000668021, S000869002, and S000970044), and 1 *Mangroveibacter plantisponsor* strain (accession number S000893171). Considering that the numbers of *Acidithiobacillus* sp. and *Pseudomonadales* strains detected represent just 1.15% and 0.04%, respectively, of the total numbers of sequences in the database for these species, the risk of misidentification in PIF is very low. For *M. plantisponsor*, a salt-tolerant nitrogen-fixing bacterium (N. Ramesh Kumar and S. Nair, unpublished data), there is a lack of information about its morphology and its ecological reservoirs. Moreover, none of these species, with the possible exception of some *Pseudomonadales* species, are expected to contaminate infant formula (10, 13, 45).

Protocol optimization and autofluorescence-related factors.

Protocols were developed both for hybridization on slides, based on the method described by Guimarães et al. (15), and for hybridization in suspension, as reported by Perry-O' Keefe et al. (35). However, the following modifications of the hybridization and fixation steps were necessary to obtain successful hybridization. Inclusion of a 10-min paraformaldehyde immersion step before ethanol fixation as a means of increasing the signal-to-noise ratio was critical and also appeared to result in a reduction in sample autofluorescence. No differences in signal intensity were found with various ethanol concentrations (50 and 80%) in the fixation step. Hybridization and washing temperatures were also modified as the highest signal-to-noise ratio was obtained at 57°C (see Fig. S1 in the supplemental material). Hybridization times of 30, 45, 60, and 90 min were found to be equally efficient. However, as autofluorescence appeared to increase with hybridization time, the 30-min assay was used preferentially. All these observations were applicable for hybridizations performed on slides and for hybridizations performed in suspension.

After hybridization, samples were stored at 4°C in the dark for a maximum of 24 h before microscopy. Visualization by microscopy was performed using an Olympus BX51 (Olympus Portugal SA, Porto, Portugal) epifluorescence microscope equipped with a filter sensitive to the Alexa Fluor 594 signaling molecule attached to PNA probe (excitation wavelengths, 530 to 550 nm; barrier wavelength, 570 nm; emission long-pass wavelength, 591 nm). To ensure that the signal obtained was not related to autofluorescence, all samples were visualized with other available filters. For every experiment, a negative control was performed simultaneously, using all the steps for standard hybridization but without addition of the probe to the hybridization solution.

***Cronobacter* probe specificity test.** Following optimization of the hybridization conditions, the specificity of the PNA probe was tested using 51 *Cronobacter* strains (representative of the phenotypic and genetic diversity in this genus), 23 *Enterobacter* strains, and 31 strains of other related bacteria or expected contaminants of milk and milk products (Table 1). The bacterial strains used were kept at -80°C and subcultured once on tryptic soy agar (VWR, Portugal) at 37°C for 24 h before each

TABLE 1. Results of the *Cronobacter* probe specificity test

Microorganism(s) (no. of strains tested) ^a	Results of FISH with PNA
<i>Cronobacter sakazakii</i> (36), including ATCC 29544 ^T and 274 ^b	+
<i>Cronobacter dublinensis</i> subsp. <i>lactaridi</i> (2), <i>Cronobacter dublinensis</i> subsp. <i>lausannensis</i> , and <i>Cronobacter dublinensis</i> subsp. <i>dublinensis</i>	+
<i>Cronobacter muytjensii</i> (3), including ATCC 51329 ^T	+
<i>Cronobacter malonaticus</i> (6)	+
<i>Cronobacter</i> genomospecies 1	+
<i>Cronobacter turicensis</i>	+
<i>Enterobacter helveticus</i> (8)	-
<i>Enterobacter pulveris</i> (6)	-
<i>Enterobacter asburiae</i> (2)	-
<i>Enterobacter hormaechei</i> (2)	-
<i>Enterobacter turicensis</i> (2)	-
<i>Enterobacter cloacae</i>	-
<i>Enterobacter aerogenes</i> ATCC 12048 ^T	-
<i>Enterobacter amnigenus</i> ATCC 33072 ^T	-
<i>Shigella flexneri</i> ATCC 12022 ^T	-
<i>Staphylococcus aureus</i> ATCC 12600 ^T , ATCC 6538 ^T , and ATCC 13565 ^T	-
<i>Staphylococcus epidermidis</i> ATCC 35983 ^T , ATCC 35984 ^T , ATCC 1798 ^T , and ATCC 14990 ^T	-
<i>Escherichia coli</i> ATCC 25922 ^T , N5, ^b and N9 ^b	-
<i>Salmonella enterica</i> serotype Enteritidis ATCC 13076 ^T , 349, ^b 355, ^b 357, ^b A1, ^b CC, ^b Sal4, ^b and Sal6, ^b <i>Salmonella enterica</i> serotype Heidelberg 354, ^b and <i>Salmonella enterica</i> serotype Typhimurium NCTC 12416 ^T	-
<i>Pseudomonas fluorescens</i> ATCC 13525 ^T and N3 ^b	-
<i>Pseudomonas aeruginosa</i> ATCC 10145 ^T	-
<i>Serratia plymuthica</i> F4 ^b	-
<i>Listeria monocytogenes</i> 747, ^b 925, ^b 930, ^b 994, ^b and 1562 ^b	-
<i>Bacillus cereus</i> ^b	-

^a T = type strain.

^b Isolate.

experiment. As shown in Table 1 and as expected from the predicted sensitivity value, SakPNA971 hybridized with all the *Cronobacter* strains, whereas no hybridization was observed for the other species used, so the practical levels of sensitivity and specificity were 100%.

Detection of *Cronobacter* in PIF. The FISH procedure using PNA was then adapted to detect *Cronobacter* directly in a commercially available PIF, based on the previously work of Mohan Nair and Venkitanarayanan (31). Initially, we performed an experiment to assess the experimental detection limit of FISH with PNA. For this, *Cronobacter muytjensii* ATCC 51329 was resuspended in reconstituted PIF (10% [wt/wt]; NAN 1 Premium; Nestle) at concentrations ranging from 1×10^8 to 1×10^2 CFU/ml. The PIF was reconstituted in water at ca. 60°C, a temperature commonly used for rehydration (13). One-milliliter aliquots of each dilution were concentrated by centrifugation as described above, and hybridization was performed in suspension or on glass slides. Microscopic visualization showed that this procedure could detect the pathogen at a concentration of 1×10^6 CFU/ml.

The theoretical detection limit was also calculated based on the assumption that for trustworthy analysis each microscopy field should contain at least five cells. This calculation considered the microscopic field area (0.0158 mm²), the slide well area (200.96 mm²), and the dilution used during the proce-

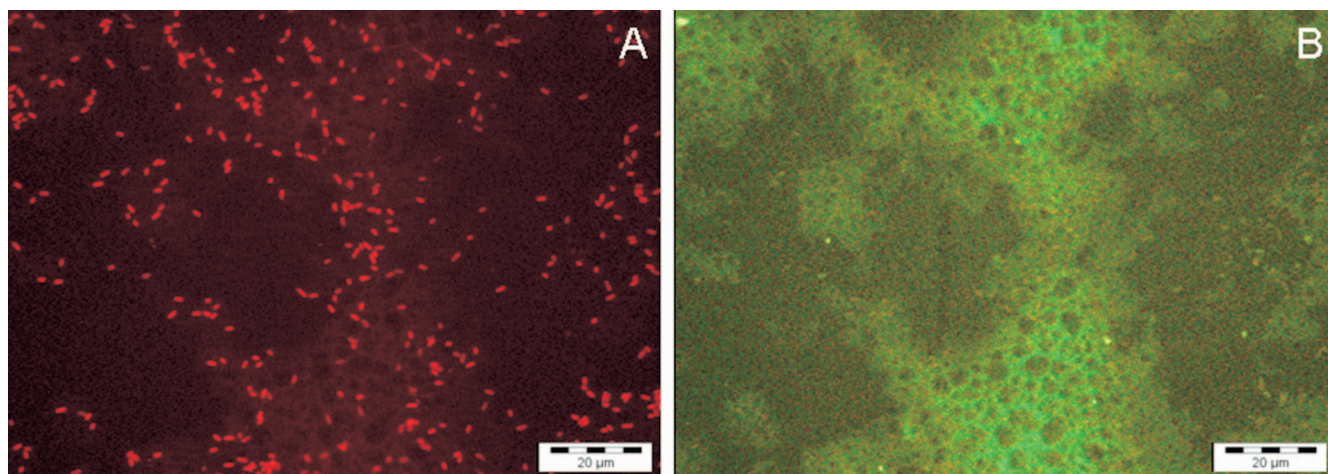


FIG. 1. (A) Detection of *C. sakazakii* 274 on a glass slide using the SakPNA971 probe, a preenriched culture (10% PIF), and an initial concentration of 1 to 3 CFU/100 ml reconstituted PIF. (B) Visualization of the same microscopic field with the green channel (negative control), showing autofluorescence of infant formula proteins and the absence of fluorescent cells.

ture. The value obtained, 2×10^5 cells/ml, was lower than the value determined by laboratory testing. The difference could be explained by the fact that the theoretical value is between the 10-fold dilutions analyzed, 1×10^2 to 1×10^8 CFU/ml. Moreover, during laboratory testing some cells might be lost in the centrifugation steps, decreasing the real cellular concentration.

After determination of the detection limit, we used the FISH procedure with PNA to detect *Cronobacter* in PIF samples with different levels of contamination. Serial 10-fold dilutions of *Cronobacter* sp. strain ATCC 51329 were prepared using reconstituted formula to obtain final concentrations of *Cronobacter* ranging from 1×10^{-4} to 1×10^7 CFU/ml (corresponding to 1×10^{-2} to 1×10^9 CFU/10 g). Given the short lag time and the growth rate of *Cronobacter* in PIF, it was expected that a single cell could produce a concentration of more than 1×10^7 CFU/ml in an 8-h enrichment period (19, 22). For this reason, after 8 h of enrichment at 37°C, 1-ml samples were taken and diluted 1:10, and hybridization was performed in suspension or on glass slides, as described above. A noncontaminated culture was prepared in parallel and exposed to the same conditions. This experiment was performed three times and was repeated with two other *Cronobacter* strains (ATCC 29544 and 274). The bacterial concentration in each enriched culture was determined by conventional counting and by FISH with PNA (see Table S1 in the supplemental material). Quantification by FISH with PNA was obtained by epifluorescence microscopy by counting a total of 15 fields. Approximately 57 to 87% of the PNA-FISH-labeled cells were detected by culture methods, and the final concentrations were always higher than the theoretical or experimental detection limit previously determined.

The procedure was able to detect *Cronobacter* in PIF samples with an initial concentration of 1×10^{-2} CFU/ml (and even with an initial concentration of 1×10^{-3} CFU/ml for 4 of 27 replicates) after an 8-h preenrichment step. The previously reported levels of *Cronobacter* in PIF samples are very low. For instance, Muytjens et al. examined 141 different powdered formulas and isolated *Cronobacter* at levels ranging from 0.36

to 66 CFU per 100 g (33). In other work, Simmons et al. isolated 8 *Cronobacter* CFU per 100 g from PIF associated with an outbreak of clinical illness in Tennessee (39). Based on our findings, our assay is capable of detecting less than 1 CFU per 10 g of PIF, which compares well with previous reports.

Autofluorescence of infant formula proteins remained detectable, but, as the sample was diluted at least 1:10 before hybridization, autofluorescence did not interfere with bacterial detection (Fig. 1). Moreover, when hybridization was performed in suspension, autofluorescence was almost undetectable.

The experiments with PIF were repeated with the same strains using freeze-dried *Cronobacter* cells, as in the experiments performed previously by Iversen and Forsythe (18). With this approach we intended to investigate if the physiological state of the cells and contamination in powdered or reconstituted infant formula could affect the outcome of FISH with PNA or the concentration determined. The results showed that there was no difference between the two physiological conditions tested.

Detection of *Cronobacter* in a mixed population. SakPNA971 was also employed with a mixed population containing *Cronobacter* cells together with other species. For this experiment, serial 10-fold dilutions of *Cronobacter* sp. strain ATCC 51329 were made in reconstituted formula, as described above, resulting in concentrations ranging from 10^3 to 10^8 CFU/ml, and mixed with *Bacillus cereus* at 10^8 CFU/ml. One-milliliter samples were concentrated by centrifugation ($10,000 \times g$ for 5 min), and hybridization was performed as described above. The sample was then counterstained with 20 μ l (10 μ g/ml) of 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and incubated for 10 min in the dark. The excess DAPI was removed, and the sample was allowed to air dry, mounted with nonfluorescent immersion oil (Merck), and covered with a coverslip. The same procedure was repeated with other species, including *Salmonella enterica* serotype Enteritidis ATCC 13076 and *Pseudomonas aeruginosa* ATCC 10145, and also with all four species simultaneously. *Pseudomonas* and *Bacillus* species were chosen because they are common contaminants of milk products and infant foods (4, 10, 13, 14, 37, 43), and *S. enterica* was chosen

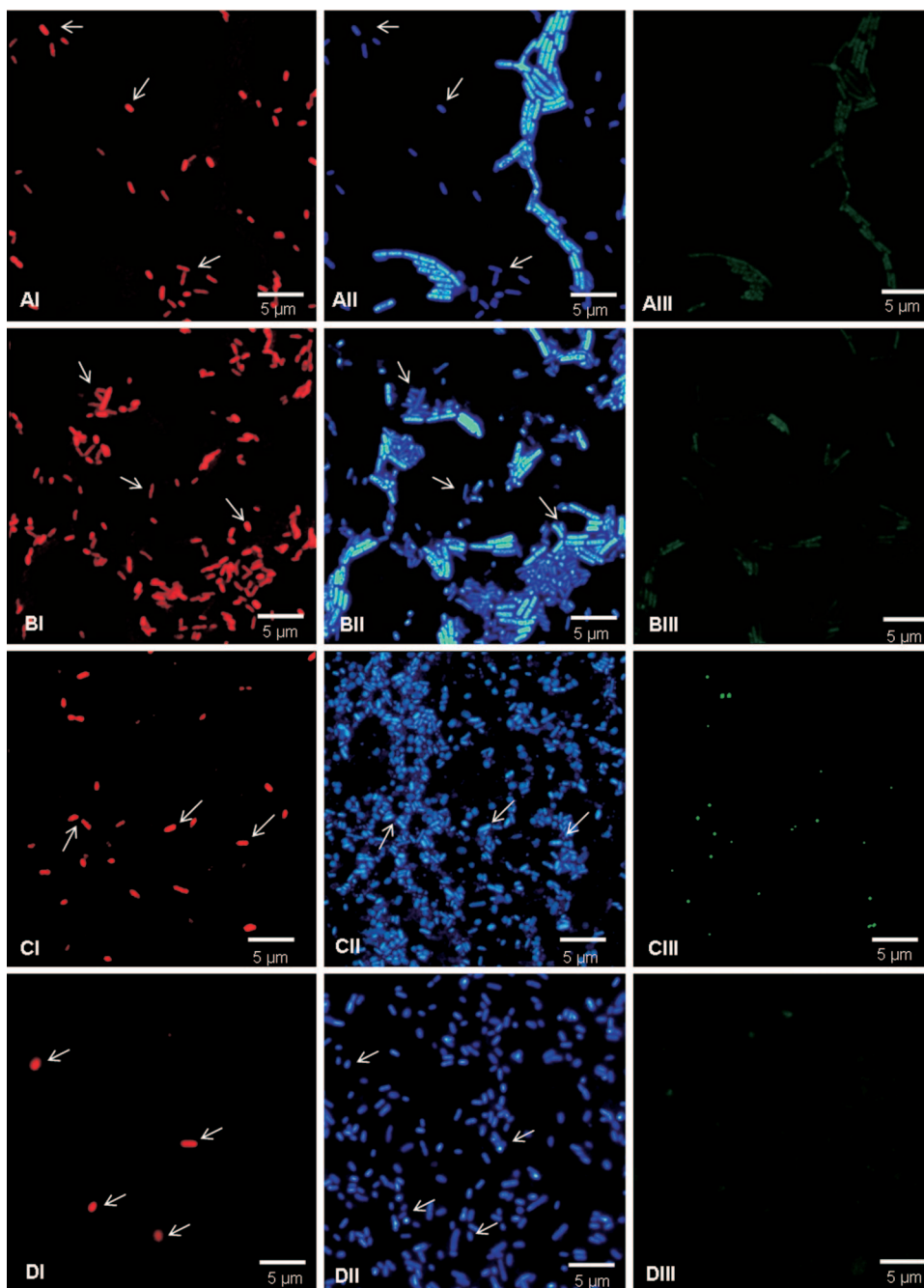


FIG. 2. Detection of *C. sakazakii* ATCC 29544 in suspension with a mixed population using SakPNA971. (A) Reconstituted PIF with *C. sakazakii* and *B. cereus*. (B) Reconstituted PIF with *C. sakazakii*, *B. cereus*, *P. aeruginosa* ATCC 10145, and *S. enterica* serotype Enteritidis ATCC 13076. (C) Reconstituted PIF with *C. sakazakii*, *S. enterica* serotype Enteritidis ATCC 13076 (100-fold concentrated), and *P. aeruginosa* ATCC 10145 (100-fold concentrated). (D) Reconstituted PIF with *C. sakazakii* and *S. enterica* serotype Enteritidis ATCC 13076 (100-fold concentrated). (Panels I) Detection of *C. sakazakii* using the red fluorescent SakPNA971 probe. (Panels II) Counterstaining with DAPI (total population). (Panels III) Visualization of the same microscopic field with the green channel (negative control). The arrows indicate *C. sakazakii* cells that could easily be visualized in the DAPI channel with the red-labeled SakPNA971 probe. All images were obtained using the same exposure time.

because it is a pathogen that is phylogenetically more closely related to *Cronobacter* that has also been reported to be an occasional contaminant of PIF (6, 7, 42, 44).

DAPI counterstaining facilitated visualization of the total bacterial population and allowed determination of the percentage of *Cronobacter* cells in the heterotrophic mixture of cells.

It was observed that FISH with PNA allows easy detection of the target microorganism in mixed populations, even when the concentration of the target microorganism is clearly lower (Fig. 2). In mixed samples containing *Cronobacter* cells that were 10- to 100-fold diluted compared to the *P. aeruginosa*, *B. cereus*, and *S. enterica* cells, *Cronobacter* cells were easily detected

using the SakPNA971 probe. Even when the proportion of *Cronobacter* cells was 1:300 (ratio of *Cronobacter* cells to *P. aeruginosa* cells to *B. cereus* cells to *S. enterica* cells, 1:100:100:100), *Cronobacter* cells were easily detectable. For samples containing *Cronobacter* cells diluted more than 1,000-fold, detection was not possible as the number of cells was below the detection limit of FISH with PNA established previously.

In conclusion, FISH procedure using the SakPNA971 probe has been shown to be a sensitive and specific method for detection of *Cronobacter* in PIF. Using this approach, a qualitative assay detected *Cronobacter* in less than 12 h, and the detection limit was less than 1 CFU per 10 g of PIF.

It is also important to note that most previously described protocols that use a molecular approach to detect *E. sakazakii* are PCR-based protocols that target DNA (23, 26, 28, 29, 31). The previous studies reported development of several primers and DNA probes, but a DNA extraction step is required and, when low levels of *Cronobacter* are present, an additional enrichment step is also needed. The total time required for the FISH assay using PNA is similar to or less than the times reported for PCR-based methods (9, 28, 29, 31), while culture-based techniques and biochemical tests, which are currently used in hospitals, take about 5 to 7 days to confirm the presence of the organism (9, 31). FISH using PNA is likely to be easily adapted for identification and quantification of *Cronobacter* in several other types of samples, such as cerebrospinal fluid and blood (17, 40, 46), although this was not tested in this work.

Future work could take advantage of suitable PNA probes that already have been developed and the very narrow emission band of the Alexa fluorophore attached to SakPNA971 to develop multiplex assays that detect a large number of different pathogens. Moreover, combination with flow cytometry, as reported by Hartmann and coworkers, could result in easier, faster, and accurate distinction of several species simultaneously (17).

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