Innovations in Dairy

October 2002

DAIRY INDUSTRY TECHNOLOGY REVIEW

Dairy Product Safety: Rapid Test Methods for Pathogens and Allergens

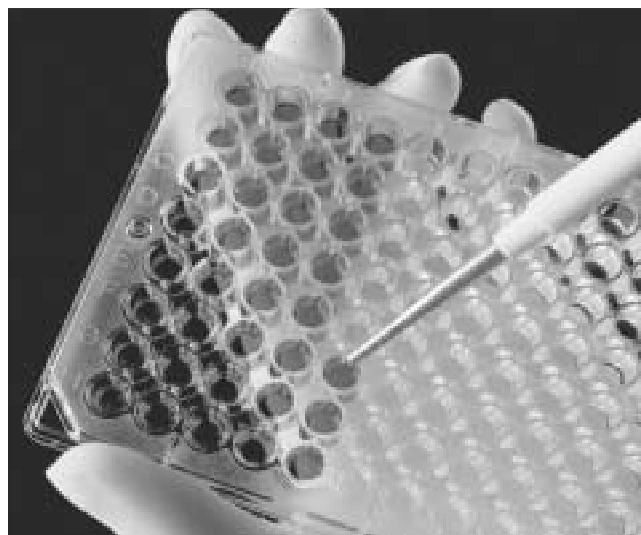
Executive Summary

New rapid testing techniques for pathogens and allergens are helping to keep dairy products among the safest foods available to U.S. consumers. This technical bulletin discusses updated information pertaining to rapid test methods for pathogen screening based on adenosine triphosphate (ATP) monitoring, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and other methods. In addition to new test procedures, another useful tool to assist dairy quality control personnel with identification of pathogens is the Bacterial Strain Database developed by Cornell University researchers. This valuable database, which contains genetic fingerprints of isolated organisms, could help food processors attain quick safety-related information about an isolated microbial strain. Another food safety issue of paramount concern to dairy processors is allergen contamination. New sandwich enzyme-linked immunoassay (S-ELISA) methods have greatly simplified allergen screening of dairy products. These new rapid test methods are helping to reduce the chance of food borne illnesses and the likelihood of product recalls for the dairy industry.

Introduction

Since the advent of pasteurization, the dairy industry has been a leader in food safety and aggressively proactive in its commitment to ensure the safety of dairy products. Two areas of attention are pathogens and, more recently, allergens. Thanks to new technological advances in convenient-to-use, rapid screening tests, both of these safety issues can now be addressed as part of a total dairy quality control program.

Bacterial pathogen contamination of dairy products is usually monitored via agar plate counting techniques, which generally take from one to five days—too long to be an effective pathogen screening tool. Although numerous new rapid methods have been proposed in the past, most proved unsuitable because



they did not distinguish between viable cells and non-living cells, were subject to interferences or lacked sensitivity. Recently, improved rapid testing technologies have overcome many of these problems and promise to revolutionize the way pathogen screening is performed in the dairy industry.

Allergens are another area of food safety concern. Approximately 2 to 3% of adults and 5 to 8% of children are allergic to foods. More than six million people in the U.S. are known to have a food allergy. Food allergies are caused by proteins that can trigger an immune response in sensitized individuals.

As the number of different ingredients used in formulated foods continues to grow, it is becoming more common for dairy processing plants to handle a wider spectrum of ingredients than they did a few years ago. This has increased the likelihood of cross-contamination of products with inappropriate ingredients—i.e., ingredients that can cause allergic reactions and are not indicated on product labels. Whether it's peanuts, tree nuts, milk, eggs, wheat or soybeans, nearly every processed food has an identified allergen in it.

Of the “Big 8” foods identified by the U.S. Food and Drug Administration (FDA) as containing allergenic proteins that are estimated to cause 90% of the allergic reactions in the U.S., these six commonly are used in formulated products. The two other allergens that comprise the “Big 8” are fish and crustaceans.

An effective allergen control program involves several components: scheduling, sequencing, sanitation and testing. Production scheduling is a key part to the successful implementation of an allergen control program.

Allergen testing is the final component of the allergen control program. For example, if an eggless product is run at the beginning of a shift and previous production on the same processing line contained eggs, the first batch of eggless product should be tested with a rapid allergen screening test to monitor the efficacy of cleaning and sanitation procedures. An important advantage of the new-generation allergen testing procedures are that they provide near real-time feedback.

Types of Rapid Tests

The primary goal of rapid test methods is to provide processors with an at-line answer to the following question: Is this production lot contaminated with significant quantities of pathogens or allergens at levels high enough to cause health problems? While no rapid pathogen screening test is currently available that will answer this question with the real-time speed required by the Hazard Analysis & Critical Control Points (HACCP) program, several recently developed rapid pathogen tests show that scientists are coming closer to reaching this goal.

In the last five years, rapid screening methods have been developed for some of the most dangerous microorganisms that cause food borne illnesses—*E. coli* 0157:H7, *Salmonella*, *Listeria monocytogenes* and *Campylobacter jejuni*. In recent years, some of these rapid test methods have replaced the lengthier traditional tests. Several different strategies are being exploited in the development of rapid testing procedures. A few examples include the following:

- ***Novel media:*** New selective culture media for detecting pathogens have significantly improved testing efficiency of traditional agar plate counting methods. New chromogens and other chemicals added to the media have created a new generation of highly efficient microbiology-based plating tests. Examples of these tests, which use novel media to enable differential detection and enumeration of pathogens on the same agar plate, include BBL-ChromAgar (BD Diagnostics), FloroCult agars (Merck) and Rainbow Agar (Biolog). Another strategy that has worked well is to add an enzyme activity indicator. For example, the addition of 4-methylumbelliferyl-(beta)-D-glucuronidase (MUG) has proven to be an efficient way to detect and count *E. coli/* coliforms, *Staphylococcus aureus* and other pathogens.

On the Horizon: Emerging Microbiological Food Safety Issues

Several key issues are likely to influence the need for increased food safety vigilance and the need for rapid pathogen test methods in the next decade:

- **Globalization of the Food Supply.** The amount of imported food is increasing substantially, and this trend is likely to continue. Consistent, widespread application of food safety systems, including HACCP systems and good agricultural practices, must be encouraged for international trade.
- **Alternative Processing Technologies and Novel Foods.** Scientists continue to be challenged to adequately address all parameters associated with the introduction of a novel food or alternative processing technology. Once developed, new technologies must be appropriately used and regulated to ensure their proper application and the product’s safety.
- **Increases in Organic Foods.** The use of manure as a fertilizer for organic crops is a significant concern. Methods are needed to reduce the presence of pathogens in manure and to effectively eliminate them before they contaminate the environment and food.
- **Changes in Food Consumption.** People’s changing dietary patterns affect their risk of food borne illness. Control and prevention methods will need to be adapted to those changing dynamics.
- **At-Risk Subpopulations.** It is likely that the number of persons at higher risk for food borne disease will continue to increase with time. The population of the United States is aging. In addition, there are an increasing number of transplant recipients, people undergoing treatment for cancer, people with AIDS and others with compromised immune system function.
- **Pathogen Evolution.** Microbial evolution has always happened and will continue to occur. Improved surveillance and new genomic technologies should help in the identification of new potential food borne pathogens before they cause significant illness. Another hope for the future is a better understanding of how human actions affect food borne pathogens.
- **Integrate Food Safety System.** A farm-to-table food safety system must involve many interested parties working together toward a common goal. The challenge is to build a system that applies science in a predictable, consistent and transparent manner to enable harmonization within and between countries.

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- ***Impedance/conductance:*** When bacterial pathogens grow in food and media, they break down proteins, fats and other large relatively uncharged molecules and convert them into amino acids, fatty acids and other smaller charged chemicals as part of their normal metabolic processes. The increase in these charged chemicals boosts the ability of the media to conduct an electrical charge. As a result, significant changes in the electrical impedance, conductance and capacitance of the medium occurs as the microorganisms grow. Instruments have been designed to accurately measure minute changes in impedance and conductance at regular time intervals (e.g., six minutes); the time required for significant changes in the impedance is automatically recorded. This time period, as well as the slope of the impedance curves, can be interpreted to provide estimates of initial contamination levels, generation times and growth densities in the sample. These types of instruments have been in use for several years and are now highly automated and easy to use.

  Purnendu C. Vasavada, Ph.D., professor of food science at the University of Wisconsin-River Falls and an expert on rapid pathogen testing methods used in the dairy industry, says that impedance/conductance-based tests have been applied in the dairy industry to solve many important food safety problems. A few examples include estimation of bacteria levels in raw and pasteurized milk and dairy products, detection of antibiotics and estimation of the shelf life of pasteurized milk. Recently, a conductance technique was reported for detecting *Salmonella* and for the enumeration of *Enterobacteriaceae* in milk.

- ***Immunoassays:*** The term immunoassay encompasses a wide range of assays used to detect and quantitate antigens (foreign molecules that enter the body) and antibodies (host proteins produced in response to the presence of antigens). When properly designed, immunoassays for pathogen detection can quickly yield results that may be difficult to determine by other techniques. There are numerous ways immunoassays can be performed. One of the most popular immunoassay techniques for screening milk for pathogens and toxins is the enzyme-linked immunosorbent assay (ELISA) method. There are many types and variations of ELISA test methods. ELISA procedures have been designed to detect and quantitate levels of pathogens, toxins and enterotoxins, antibiotics, pesticide and drug residues and allergens. Some ELISA methods can be used for the detection of specific microorganisms such as *E. coli* 0157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* in milk and dairy products. Highly automated and sensitive bench-top instruments based on immunoassay methods are now available and have significantly reduced the time and labor required to obtain results. According to Vasavada, the rapidity and sensitivity of immunoassay-based test kits and systems have come a long way in the past few years due to development in immunoprecipitation devices, lateral flow devices and immunomagnetic separation (IMS) techniques. Immunoassay tests offer three important advantages: speed of analysis, sensitivity and high specificity for detecting the target pathogen.

- ***Bioluminescence:*** Adenosine triphosphate (ATP) content has been shown to be proportional to the number of viable cells present in a food sample. ATP is generally associated with living cells. Therefore, quantitating ATP levels is a way to estimate the number of viable cells in a test sample. Luminescence techniques are sensitive and allow for the rapid detection of microbial "dirt." The major problem with ATP testing is that it is a nonspecific indicator of pathogen contamination and prone to providing false positives. ATP can be measured in one to two minutes using firefly luciferase and cofactors to produce light. The light produced is sensitively and accurately measured by luminometers. Accurate detection of bacteria or other microorganisms using this technique depends on three factors: (1) the number of microorganisms present; (2) the amount of ATP per cell; and (3) the presence of other living cells such as somatic or mammalian cells. As a result, attempts to correlate ATP levels with total microbial counts may or may not be accurate because the amount of light generated depends on these two other factors. Vasavada points out that ATP levels may vary depending on the metabolic activities of the organisms and different groups of mixed microbial populations. Also, sanitizer residues that might be present in the test sample have been known to quench the ATP reaction. Despite these limitations, ATP quantitation based on bioluminescence measurements has been widely used to monitor the efficacy of sanitation in dairy plants.

- ***PCR-based methods:*** The polymerase chain reaction (PCR) technique is based on detection of specific gene fragments by in vitro enzymatic amplification of the target DNA followed by detection of the amplified DNA molecule by electrophoresis, ELISA or some other analytical technique. One particularly exciting new technology for the rapid quantitation of DNA is lab-on-a-chip technology in which electrophoretic separations of DNA molecules and detection are all performed with nanoliter quantities of sample and reagents using microfluidics technology in combination with fluorescence detection. The advantages of PCR/DNA methods are their high sensitivity and specificity.

  In the past, PCR was limited by the difficulty in obtaining specific DNA primers and production of nonspecific PCR products. Another problem was false-positives because the test was unable to differentiate DNA from dead organisms versus live, viable organisms. Recently, interest in PCR has been renewed because automation and improvements in PCR systems have addressed these shortcomings. With the strong advantages of specificity and sensitivity and improvements that have corrected many of the deficiencies, PCR is now viewed as an extremely attractive option for rapid pathogen detection.

  Twenty-five years ago, the standard technique for isolating low levels of *Listeria* in food took one month. Now, the time

frame has been reduced to two to three days, and new PCR-based systems can identify *Listeria* in as little as six hours.

North Carolina State University researchers MaryAnne Drake, Ph.D., and Lee-Ann Jaykus, Ph.D., are developing PCR tests to detect viable bacterial pathogens, including *L. monocytogenes, Salmonella, E. coli* 0157:H7 and *S. aureus* in dairy products.

One of the biggest challenges with rapid detection methods is detecting only viable cells and not dead cells. The ability to tell whether cells are alive or dead in a sample is important because live cells have the potential to grow into millions of cells in just a few hours, while dead cells can't.

By using PCR methods that are capable of amplifying specific DNA sequences millions of times in a few hours, Drake and Jaykus are able to achieve exponential amplification of specific bacterial DNA, even if only a few cells are present in the sample.

Recently, Drake and Jaykus have decided to target ribonucleic acid (RNA) sequences because they are more indicative of live cells than is DNA. RNA carries out the instructions encoded in DNA. Essentially, RNA assembles proteins, one amino acid at a time, using the sequence of nucleotides along a strand of DNA (i.e., a gene) as its guide. There is more than one type of RNA. For example, messenger RNA (mRNA) carries the genetic information out of the nucleus for protein synthesis; transfer RNA (tRNA) decodes the information; and ribosomal RNA (rRNA) is a molecular assembly involved in protein synthesis.

Initially, Drake and Jaykus studied rRNA as a possible sequence target for amplification, since they expected rRNA of dead cells to degrade after death. However, their studies revealed that rRNA, while less stable than DNA, was not an ideal target in most foods since it does remain fairly stable following cell death. Recent research findings by Drake and Jaykus, as well as other researchers, show that mRNA is very transient following cell death. Therefore, mRNA is currently being targeted in amplification experiments.

In addition to the actual detection of bacteria, Jaykus has identified a method for removing PCR inhibitors from foods in order to use a reasonable sample size in the assay. The extraction process efficiently concentrates the bacteria in dairy food samples in a manner suitable for amplification by PCR.

New Database Helps With Pathogen Identification

Another relatively new tool to help food safety personnel in early pathogen detection is the Bacterial Strain Database. The database, created by Cornell University researchers Martin Wiedmann, Ph.D., and Kathryn Boor, Ph.D., and funded by USDA and Dairy Management Inc.™, contains genetic fingerprints of isolated organisms obtained by a number of commonly used sub-typing procedures. Using this Web-based database, users can locate isolated strains that have been characterized by: (a) DNA sub-types identified through banding patterns, produced by ribotyping or by pulse-field electrophoresis; (b) DNA-sequencing of selected bacterial genes; or (c) phenotypic data such as nutrient consumption and use (e.g., carbohydrates, fat, etc.).

Strains can be compared to each other and/or to a user's new entry. In addition, a user can locate information about all entered organisms, such as origin (animal, human, food, environment), specific isolation site, year of isolation, geographic state of isolation and reported incidence of food borne illness (if any).

This kind of information can help food processors attain quick safety-related information about an isolated microbial strain—in the privacy of their own labs and without the added costs of third party investigative services. This interactive database is available at **www.pathogentracker.net**.

S-ELISA Tests For Allergen Screening

Currently, the most popular type of food allergen testing is based on sandwich enzyme-linked immunoassays (S-ELISAs). A target allergen protein is extracted from samples with a buffered salt solution. The extracted protein is sampled and added to antibody-coated microwells, where it binds to the antibody during an incubation period. After a wash step, enzyme-labeled antibody (conjugate) is added to the antibody wells and is allowed to attach to the bound allergen, forming an "antibody sandwich" around the allergen. After another wash step, substrate is added which reacts with the conjugate to produce blue color. The intensity of blue color is proportional to the amount of allergen.

A stopping reagent is added, and the color of the resulting solution is observed. The intensity of blue color is indicative of the amount of allergen present; red color indicates little to no target food allergen is present. A different kit must be used to test for each type of allergen.

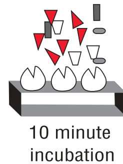
A common practice is to provide S-ELISA test kits to be used as a quick qualitative test at the quality control labs in the dairy processing plants. Samples that test positive can be retested at a corporate level laboratory equipped with a microwell colorimetric reader for accurate quantitation of the levels of allergen present. A decision can be made to ship or not to ship the batch of samples based on the level of allergen contamination.

Testing clean-in-place (CIP) solutions, final product and certain equipment after the sanitation has been completed can identify sources of cross-contact and also verify cleanliness before changeover.

It is important to realize some of the limitations inherent in this type of testing. Because high temperature may denature protein, test antibodies may not capture a sample's allergenic component. This is especially true with egg products, which denature at a relatively low time and temperature combination. Since denatured protein may remain allergenic, it is recommended that products be tested prior to baking or cooking.

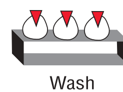
Food Allergen Sandwich ELISA Test

Step 1: Add sample extract to antibody-coated microwell.



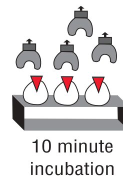
10 minute incubation

Step 2: Allow time for allergen to bind to antibody.



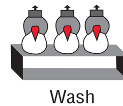
Wash

Step 3: Add conjugate.



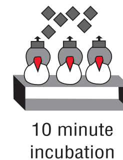
10 minute incubation

Step 4: Allow time for conjugate to attach to bound allergen. "Antibody sandwich" forms around allergen.



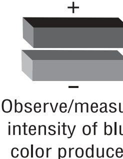
Wash

Step 5: Add substrate. Blue color forms.



10 minute incubation

Step 6: Add red stopping reagent and read results.



Observe/measure intensity of blue color produced

Legend: Antibody; Food allergen; Conjugate; Food allergen/antibody reaction; Substrate

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Another problem can occur when testing samples that have high oil content. Although low levels of protein may be present in edible oils, they may be difficult to extract with standard extraction solutions and may not be detected by the test.

Also, it is important to note that using a test kit designed for testing the presence of peanut protein is not appropriate to use for screening samples for almonds, pecans or other tree nuts. Care must be exercised when testing for egg allergens. Some test kits only test for egg white proteins; egg yolk proteins can also cause allergic reaction in people sensitized to egg yolk proteins.

Conclusion

The increase in food borne illnesses and specific types of pathogens is due to various consumer, manufacturing and regulatory trends that may set the stage for contamination or minimal screening methods. For example, there are changes in consumer consumption habits and preferences for minimally processed convenience foods, as well as an increase in certain risk groups who are most vulnerable to diseases.

Also adding to the problem are changes in food production, distribution and globalization of supply that expands the potential for imports tainted with pathogens or pesticide residues. Compounding the problem are new types of pathogens, as well as new strains of recognized pathogens, and both are appearing in food products where they have never before been identified.

It is likely that technologies based on immunoassays and PCR methods will emerge as the two favorite types of rapid pathogen screening tests for dairy products because of their enhanced specificity, sensitivity and efficiency compared to other methods. PCR tests that measure pathogen DNA or RNA are more sensitive but also are more expensive than immunoassay methods.

To continue to deliver safe dairy products to the American public, dairy processors will be increasingly dependent on new rapid, accurate, sensitive and specific screening tests for pathogens and allergens.

Editor's note: The mention of specific methods or instruments in this article does not constitute an endorsement of products or methods by Dairy Management Inc.™ To gain an understanding of the most reliable rapid test kits and methods, readers are advised to consult with regulatory agencies. One source to consider is the Association of Analytical Communities Research Institute (AOAC RI). The AOAC RI can be reached at 301-924-7090 or visit http://64.55.218.151/testkits/testkits.html. It has certified more than 40 test kits in the last decade. When test kits have been certified by the AOAC RI and received Performance Tested Methods certification, users can be assured that an independent third-party review of the kit has been made and the kit performs as claimed.

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