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Review

Challenges in Recovering Foodborne Pathogens from Low-Water-Activity Foods

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ABSTRACT

There are numerous obstacles to the detection of foodborne pathogens in foods that exhibit a low water activity (a_w). These obstacles include the presence of antimicrobial compounds, particulates, PCR inhibitors, and fatty matrices. New approaches should be sought to increase the sensitivity of pathogen testing in low-a_w foods and to overcome the effects of various inhibitors and antimicrobials. The U.S. Food and Drug Administration and other laboratories are working toward this goal. This review will address these issues while delineating specific inhibitors and antimicrobials that impede testing of low-a_w foods. A review of relevant rapid and conventional testing methodologies for *Salmonella* in low-a_w foods will also be discussed.

Key words: Antimicrobial inhibitors; Dried foods; Low-water-activity foods; *Salmonella* recovery; Spices

Foods with low water activity (a_w) are variably described as having an a_w of <0.60, <0.70, and/or <0.85 *(5, 27, 39)*. Low-a_w foods may be more appropriately defined as those with an a_w of <0.60, and intermediate a_w foods would be those with an a_w between 0.60 and 0.85. For the sake of this review, any foods with an a_w of <0.85 will be considered low-a_w foods to comport with the usage of this term in referenced citations. The inclusion of intermediate-moisture foods within the low-moisture food group is often done for convenience with respect to defining microbial growth, because the cutoff for most microbial growth occurs at an a_w of approximately 0.85. Low-a_w foods include an array of foods consumed as ready-to-eat or used as ingredients in other food products, including dried dairy products, milk powders, infant formulas, casein, whey protein concentrate, lactose, flours, pastas, infant cereals, breakfast cereals, cocoa powder, chocolates, sweets and other confectionaries, dried egg powders, dried noodles, dried soups, tea leaves, dried fruits and vegetables, nuts, peanuts, seeds, legumes, grains, herbs, spices, condiments, dried pet foods, dried meat, and fish products *(27)*. Cordier *(27)* also mentioned that some jams and jellies with an a_w of ca. 0.75 are occasionally described as low-a_w foods, although these foods are not low in moisture. It is well established that foods of both low and intermediate a_w are periodically contaminated with foodborne pathogens. The lower a_w does not prevent contamination or survival of the most common pathogens; only their growth is inhibited. For foodborne pathogens such as the Shiga toxin–producing *Escherichia coli* with a very low infectious dose, even low levels of contamination may be problematic *(78)*.

Low-a_w foods have been implicated in a large number of foodborne outbreaks and recalls. Table 1 lists outbreaks recorded between fiscal years 2002 and 2017. The U.S. Food and Drug Administration (FDA) reported that from fiscal years 2007 to 2009, imported spices harbored *Salmonella* at a 6.6% rate, which has been consistent for the past 30 years *(83)*. Dried spices are 1.9 times more likely to be contaminated with *Salmonella* than are any other FDA-regulated food import *(84)*. However, frequency of contamination is not the only issue with foodborne illnesses associated with low-moisture foods.

As noted earlier, low populations of pathogens have been associated with outbreaks, including an outbreak associated with chocolate in which only 1 to 6 CFU/g *Salmonella* resulted in illness, with an infectious dose of ca. 50 cells *(36)*. A second chocolate-associated outbreak had an estimated infective dose for *Salmonella* of $\leq$10 cells. The chocolate bars positive for *Salmonella* were said to contain $\leq$10 CFU/100 g *(51)*. A paprika potato chip–associated *Salmonella* outbreak was reported with only 0.45 CFU/g *(58)*. Low levels of foodborne pathogens in products such as dried spices may also serve as inocula in higher moisture foods if these pathogens enter the foods, postprocessing, leading to a potential for growth of the pathogen and further contamination.

Low population levels of foodborne pathogens can increase the difficulty of identifying contaminated product with respect to low-moisture foods. Low population levels

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TABLE 1. *Foodborne disease outbreaks associated with low-moisture foods, 2002 to 2017, worldwide*[a]

Year	Pathogen	Associated product	Location	Reference
2017	*Escherichia coli* O121	Flour	Canada	*2, 67*
2017	*E. coli* O157:H7	Soy nut butter	United States	*25*
2016–2017	*Salmonella* unknown serotype, *Salmonella* Enteritidis	Sesame seeds, tahini	Greece, Germany, Czech Republic, Luxembourg	*30*
2016	*Salmonella* Virchow	Organic shake and meal replacement (powder)	United States	*23*
2016	*E. coli* O121, *E. coli* O26	Flour	United States	*24*
2016	*Salmonella* Montevideo, *Salmonella* Senftenberg	Pistachios	United States	*21*
2015	*Salmonella* Paratyphi B variant	Raw sprouted nut butter	United States	*22*
2014	*Rhizopus oryzae*	Dietary supplements, Solgar ABC Dophilus powder	United States	*81*
2014	*Salmonella* Braenderup	Nut butters (almond, peanut)	United States	*19*
2014	*Salmonella* Stanley	Cashews (cashew cheese)	United States	*20*
2014	*Salmonella* Newport, *Salmonella* Oranienburg, multiple other serotypes	Chia powder	United States, Canada	*42*
2013	*Salmonella* Montevideo, *Salmonella* Mbandaka	Tahini sesame paste	United States	*18*
2012	As above, multiple serotypes	Tahini sesame paste	New Zealand	*63*
2012	*Salmonella* Bredeney	Peanut butter	United States	*16*
2012	*Salmonella* Infantis	Dry dog food	United States, Canada	*17*
2011	*E. coli* O157:H7	In-shell hazelnuts	United States	*14*
2011	*Salmonella* Enteritidis	Turkish pine nuts	United States	*15*
2011	*E. coli* O157:H7	Raw shelled walnuts	Canada	*7*
2010	*Bacillus cereus*	White pepper	Denmark	*80*
July 2009–Apr. 2010	*Salmonella* Montevideo, *Salmonella* Senftenberg	Black and red pepper	United States	*32*
2009	*E. coli* O157:H7	Raw cookie dough	United States	*12*
2009	*Salmonella* Montevideo	Pistachios	United States	*13*
2008–2009	*Salmonella* Typhimurium	Peanut butter	United States	*8*
Dec. 2008–Apr. 2009	*Salmonella* Rissen	White pepper	United States	*80*
2008	*Salmonella* Typhimurium	Raw wheat flour	New Zealand	*60*
2008	*Salmonella* Kedougou	Infant formula	Spain	*70*
2008	*Salmonella* Agona	Rice and wheat cereal	United States	*11*
Mar. 2007–Sep. 2008	*Salmonella* Senftenberg	Fennel seed	Serbia	*47*
Aug. 2006–Feb. 2007	*Salmonella* Tennessee	Peanut butter	United States	*9*
2007	*Salmonella* Schwarzengrund	Dry pet food	United States	*3*
2007	*B. cereus*	Spice blend	France	*80*
June 2007–Sep. 2007	*Salmonella* Wandsworth, *Salmonella* Typhimurium	Seasoning, snack puff	United States	*10*
Oct. 2002–July 2003	*Salmonella* Agona	Anise seed	Germany	*54*
Aug. 2002	*Salmonella* Braenderup	Curry powder	United Kingdom	*80*

[a] Adapted from Gurtler and Keller *(40)*.

may be below detection limits for most current detection methods, which typically rely upon small composite sample sizes *(79)*. In addition, numerous antimicrobials, inhibitors, and other confounding factors increase the difficulty of the detection of foodborne pathogens. In particular, spices may present unique challenges in the detection of foodborne pathogens. For some spices, the only means of eliminating the effects of antimicrobials on detection is by further dilution of samples, resulting in a decrease in the limit of detection *(79)*. In this review, the difficulties associated with low population levels, low frequency, and the presence of antimicrobial compounds will be discussed.

NATURAL ANTIMICROBIALS IMPEDING PATHOGEN DETECTION

Detecting and isolating bacteria from low-a_w foods, particularly spices, has proven to be challenging due to the inherent antimicrobial nature of many spices, such as cinnamon, cloves, allspice, oregano, thyme, garlic, onion, mace, nutmeg, turmeric, black pepper, bay leaf, rosemary, white pepper, sage, and marjoram. Many of the antimicrobials in the essential oils of spices are composed of phenolic constituents *(4, 28, 43, 74)*. In addition, antimicrobial compounds in plants and spices also include organic acids,

methylated flavones, flavanols, alkaloids, glucosides, glycosides, and dienes *(4, 34, 44, 62, 75, 86)*. Examples of antimicrobial constituents in spices are allicin in garlic and onion, allylisothiocyanate in mustard, cinnamaldehyde in cinnamon, eugenol in cloves and allspice *(28),* and carvacrol and thymol, which are constituents in the essential oils of both thyme *(49)* and oregano *(74)*. The most potent antimicrobials in spices are essential oils and phenols, followed by aldehydes, ketones, alcohols, and sulfur-containing compounds in seeds *(27)*.

It is generally recognized that gram-positive bacteria are more sensitive to the essential oils in spices than are gram-negative bacteria *(26)*. Because of the antimicrobial nature of many spices, higher dilutions are often used in detection methods; lower dilutions often contain high enough concentrations of essential oils to inhibit bacterial colony formation and recovery by enrichment *(43)*. The challenge is in diluting the spices sufficiently to reduce toxicity without undermining the statistical validity of the sampling regimen.

Despite the presence of antimicrobial compounds in most spices, not all spices are the same, and the quantity and type of antimicrobial compounds present do not always repress microbial growth, particularly the growth of some pathogens such as *Salmonella*. One example is black pepper, which can actually support the growth of *Salmonella (53)*. Consequently, the FDA *(82)* recommends that many spices, such as black pepper, celery seed or flakes, chili powder, cumin, paprika, parsley flakes, rosemary, sesame seed, and thyme, be diluted 1:10 with tryptic soy broth (TSB). There is, however, a recommended 60-min soak time, presumably for the resuscitation of injured cells that may be present in a low-moisture product, prior to enrichment and plating. A similar 60-min soak period is recommended for other dried products such as prepared powdered mixes. In other spices, such as dried garlic flakes or onion flakes and powder, the FDA recommends the addition of 0.5% K_2SO_3 to neutralize the antimicrobial toxicity *(43)*. However, for allspice, cinnamon, cloves, and oregano, the FDA *Bacteriological Analytical Manual* (BAM) *(82)* states that there is no known procedure for neutralizing the antimicrobial properties of these spices during sampling. *Salmonella,* in a physiologically or metabolically dormant state, while desiccated, is less susceptible to the antimicrobial essential oils in spices; nonetheless, when spices are diluted for enrichment, *Salmonella* becomes much more susceptible to the antimicrobial constituents, impeding detection *(64)*.

The FDA categorizes spices as category I or II foods, which typically do not undergo a lethal inactivation step for *Salmonella* prior to human consumption. For category I spices (intended for sensitive populations), the FDA requires 60 random samples from each lot with 15 25-g samples pooled into each of 4 375-g composites (1,500 g total) *(79)*. Category II spices (intended for consumption by the general population) requires 30 samples, with 25 g from each sample pooled into two 375-g composites (750 g total) *(79)*. Thus, to sample a category II food (with a 1:10 dilution), two 375-g composites would be added to two 3,375-mL volumes of presterilized TSB and incubated for 24 h at 35 ± 2°C. Further, to sample a category I food, four 375-g composites would be added to four 3,375-mL volumes of TSB and incubated for 24 h at 35 ± 2°C.

Nevertheless, the FDA recommends diluting clove samples 1:1,000 (to overcome the antimicrobial constituents in cloves, which would impede detection of *Salmonella*) *(82)*. Smaller amounts of the spice could be tested to lower the volume of diluent, but that could adversely affect the sensitivity of the test.

By means of illustration, an industry representative *(55)* reported involvement with testing a 0.20 a_w product by testing 30 75-g samples, which elicited an overall incidence of 50% contamination with *Salmonella*. At one point, a decision was made to begin testing 30 25-g samples, which represented a 66% reduction in sampling volume. The results were that detection of *Salmonella* in the product dropped from 50% to only 25%, demonstrating the precipitous reduction in detection sensitivity when sample volume is reduced. Clearly, new methods are needed to increase sample size and sensitivity when analyzing products such as cloves, cinnamon, oregano, and allspice, which contain higher concentrations of antimicrobial components.

Pathogens, when present in spices, are often found at very low levels and require a preenrichment step to increase populations to a detectable level *(43)*. A 1:100 dilution in TSB is appropriate to preenrich cinnamon, oregano, and allspice for the recovery of *Salmonella (43, 82)*. Incubating cloves in TSB at 35°C overnight releases the antimicrobials eugenol and methyl ether cineol into the enrichment culture, impeding pathogen recovery *(43)*. Hence, the recommendation of 1:1,000 dilution for cloves. The sensitivity of any enrichment assay is essentially 33% of its weight in grams. Hence, an enrichment approach that tests 4×375 g (1,500 g) from a 60-sample composite provides a sensitivity of ≥ 1 CFU/500 g with 95% confidence *(31, 61)*. Thus, a 1:1,000 dilution of a 25-g sample would provide a sensitivity of ≥ 1 CFU/0.0083 g or ≥ 120 CFU/g.

Other researchers have also found different levels of dilution required during preenrichment for effective recovery of *Salmonella*. Graubaum et al. *(35)* determined that basil, galangal, ginger, garlic, peppermint, oregano, clover, and mustard seeds required a 1:100 dilution for effective recovery of *Salmonella,* whereas oregano and cinnamon required a 1:1,000 dilution. However, only a 1:20 dilution was needed for recovery of sublethally injured *Salmonella* from 26 herbs and spices, including pepper and paprika.

Rather than the typically used method of dilution to reduce antimicrobial content of some spices, some research has been focused on direct elimination or reduction of the antimicrobial activity of many spices. One example is work by Zhang et al. *(89),* who evaluated a new shake and decant method for sampling cloves for *Salmonella* and compared this method with the standard BAM method. In their procedure, artificially inoculated cloves were diluted 25 g into 225 mL of TSB, shaken for 60 s, and then decanted, and the medium was incubated for 24 h at 35°C. The new method was compared with the BAM direct enrichment method in which the 25 g of cloves was preenriched in 225 mL of TSB overnight. The newly tested method resulted in positive results for 99 (82.5%) of 120 samples, whereas the

BAM method resulted in positive results for only 31 (25.8%) of 120 samples. Further, Zhang et al. *(89)* tested the newly developed method against the Pathatrix system, a system that includes use of immunomagnetic beads to sequester pathogens as a means to improve testing efficacy. It was determined that plating the immunomagnetic beads on xylose lysine deoxycholate (XLD) agar from the preenrichment, prior to incubation, attained the same results as the overnight preenrichment method (positive results for 99 of 120 samples; 82.5%). The authors concluded that the new shake and decant method or the Pathatrix direct plating method could be used with equivalent results.

RECOVERY OF DESICCATION-INJURED PATHOGEN CELLS

Another challenge to recovering pathogens from low-a_w foods is the stress (sublethal injury) the bacteria incur during the desiccation process, which may preclude recovery on standard nonselective or selective media. For this reason, nonselective preenrichments at temperatures slightly less than the optimal growth temperature of the pathogen may assist in reviving injured cells *(85)*. Further, drying the foods, which lowers the a_w, leads to sublethally injured cells, which are, very often, difficult to recover as viable CFUs *(43)*. As noted earlier, the FDA recommends a 60-min soaking period prior to further analysis. Additional methods have been examined for their ability to improve recovery of injured cells, including the addition of sodium pyruvate, catalase, superoxide dismutase, Oxyrase, etc. *(41, 85)*. Additionally, overlay and underlay methods have been used with success to allow injured cells the needed time for recovery prior to the absorption of inhibitory compounds through a nonselective thin agar layer *(50, 88)*. Although multiple methods exist, no real consensus exists for the best means of recovering injured cells.

When using large preenrichments >1 L, it is recommended that they be prewarmed prior to inoculation and incubation because it may take several hours for 3,750 mL of enrichment broth (as used for a 375-g sample) to reach a target incubation temperature *(43)*. It may also be necessary to enrich spices known to contain antimicrobial essential oils for a full 24 h rather than using a shorter recommended incubation period *(43)*. Certain peppers are acidic and, therefore, also require a pH adjustment of the medium toward neutrality. The same principle should be applied to spice blends when testing for pH prior to enrichment and incubation, because their components may overcome the buffering effect of buffers commonly present in media used for preenrichment.

OTHER ISSUES IMPEDING ACCURATE DETECTION AND ENUMERATION OF FOODBORNE PATHOGENS IN LOW-A_W FOODS

Additional logistical problems can also occur when working with dried products. One example is leafy herbs, which have been found to have high water absorption rates and corresponding lower density *(43)*. Dilution of such products at only 1:10 results in an overly viscous mass; consequently, a 1:20 dilution is recommended. Some seed products, such as chia or flax seeds, also require a 1:20 dilution to prevent gelling. Further, Keller et al. *(52)* noted that peanut butter is also very difficult to plate and enumerate at dilutions as low as 1:10 and 1:100. Needless to say, all additional dilutions made to assist in the logistical effort required in sample analysis may reduce the overall sensitivity of the analysis itself.

Gums are inherently difficult to sample without increased dilutions because of their gelling and thickening ability, entrapping the bacterial cells in the gum matrix *(43)*. Dilutions must often be made up to 1:100 because 1:10 and 1:50 dilutions still may be too viscous; thus, larger plating volumes are often required *(43)*. Carrageenan and locust bean gums require 1:100 dilutions, and many other gums may be diluted 1:50 *(43)*. The BAM methods for *Salmonella* recommend adding 1% cellulase to lower the viscosity and release entrapped *Salmonella* cells from guar gum, followed by holding at room temperature for 60 min and then incubating at 35°C for 24 h *(82)*.

Cocoa and other chocolate products pose problems in sampling due to the dark coloration on media, which masks or precludes accurate readings. Preenrichments are recommended for *Salmonella* testing in chocolates to overcome issues with natural pathogen growth inhibitors *(76)*. Other sampling issues with confectionaries include low pH in fruit jellies and alkaline pH in "dutched" cocoa powder. It has also been assumed that casein (via milk added in the BAM method) should be added to enrichments to neutralize polyphenols that may inhibit recovery of salmonellae from chocolate and cocoa products *(82)*. There was some support for this method in the isolation of *Salmonella* from a published patent *(72)*. The casein was believed to neutralize PCR inhibitors in chocolate.

Another logistical difficulty is obtaining appropriate sample sizes. For example, problems exist in cutting pet treats, such as baked biscuits, bones, dried ears and hooves, and pet chews, into the appropriate sampling sizes for use in detection methods. For this reason, it is recommended that the entire pet treat be sampled either by sampling the rinsate or enriching the entire sample in broth. In addition, some food matrices must be broken up enzymatically or mechanically prior to testing, such as with pasta noodles, which need to be chopped or ground into a fine powder to liberate cells. Nut meats and dried fruits may have internalized bacteria; hence, the BAM recommends blending for 2 min *(82)*. When sampling powders, suspension dilutions must be plated immediately to prevent powder from settling out and skewing results when bacterial cells are not uniformly distributed prior to plating. Another confounding issue related to particulates is that they may resemble growth on plating media. Quantitative methods can prove problematic in determining how to distinguish between a colony and a particulate for plate counts or coliform counts. Furthermore, colonies that are too numerous or not well separated on agar plates can sometimes appear as tiny bubbles when testing powders and, hence, are recorded as <10 CFU *(55)*.

Seemingly benign components of media or components in the food samples can also negatively impact identification and recovery. Triphenyl tetrazolium chloride added to some

media to illicit a color change with bacteria has been reported to be slightly antimicrobial and could skew the aerobic plate count, especially in cases of injured cells *(55)*. With *Salmonella,* the microbiota can sometimes change the pH of XLD agar, which can inhibit H_2S production and affect identification. Some low-a_w foods such as jams have low pH, which must be neutralized. High-fat matrices often entrap cells; thus, the use of lecithin or other nonantimicrobial surfactant is helpful in releasing cells from the matrix.

An additional issue frequently overlooked with low-a_w products is that many substrates, such as black pepper, are hygroscopic in nature and, if not stored in hermetically sealed containers, can absorb moisture, equilibrating with ambient humidity and potentially promoting bacterial growth *(64)*. An $a_w > 0.85$ permits growth of *Staphylococcus aureus,* which has been linked to outbreaks of foodborne illnesses *(27)*. Care must be exercised during handling to ensure the integrity of a low-moisture product and to ensure that assumptions with respect to stability do not compromise testing.

ADVANCED TESTING METHODS

Current official cultural testing methods such as those published in the BAM all have similar negative aspects with respect to time and resources required to accomplish their expressed goals. More recently, researchers have gravitated to the use of newer molecular or immunological techniques in an effort to reduce time and labor as well as to increase the efficiency of detection.

Wang et al. *(84)* evaluated a number of parameters to determine whether other methods were superior to the current BAM culture method for detection of *Salmonella* in pine nuts, which were implicated in a 2011 outbreak. The BAM method utilizes lactose broth (LB) as a preenrichment medium. They also tested the Pathatrix Auto, VIDAS Easy SLM, and two quantitative PCR (qPCR) methods in addition to the BAM culture method. The two qPCR methods were evaluated in LB, buffered peptone water (BPW), modified BPW (mBPW), universal preenrichment broth (UPB), and BAX MP medium. Wang et al. reported no differences among the five preenrichment media using the BAM culture method. The two qPCR methods returned a higher number of false-negative results in 24-h cultures preenriched with LB than in cultures preenriched with the other four media, and results were equivalent to the culture method in BPW, mBPW, UPB, and BAX media. The VIDAS Easy and qPCR were similar, and the Pathatrix method was least effective. The Automatic PrepSEQDNA extraction, using 1,000 mL of preenrichment, was as effective as manual extraction methods.

Caballero et al. *(6)* compared the ANSR *Salmonella* test and the Performance Tested Method (based on the nicking enzyme amplification reaction isothermal nucleic acid amplification technology) with the BAM culture method for detection of low levels of *Salmonella* in dried pet food, black pepper, almonds, peanut butter, cocoa powder, and soy flour. These authors reported no significant differences in the number of positive results obtained.

Ryan et al. *(73)* tested four rapid molecular-based detection assays for the presence of 68 individual strains of *Salmonella* in pet food and dark chocolate. Strains of *Salmonella* in low-a_w foods may be sublethally injured or have low populations that are often difficult to detect; consequently, false-negative rates can frequently run >10%, proving a serious challenge for this method. The four assays tested were assay A, an isothermal nucleic acid amplification assay; assays B and C, two PCR-based assays; and assay D, an assay that detects bacterial surface molecules. Because assay C poorly detected three *Salmonella* strains not belonging to *Salmonella enterica* subsp. *enterica,* assay C was eliminated from further study. False-negative results for dry pet food were 9% for assay A, 4% for assay B, and 26% for assay D. False-negative results for dark chocolate were 12% for assay A, 16% for assay B, and 15% for assay D. Hence, greater attention should be dedicated to studies addressing improved preenrichment and selective enrichments for these low-a_w foods.

Other researchers have made an effort to address enrichment and preenrichment conditions in order to improve testing methods. Lahti et al. *(57)* determined that enrichment of dry fermented sausages (after −70°C freezing and thawing) for 18 to 24 h was more effective than 6-h enrichments in modified TSB at 41.5°C for the recovery (on traditional agar media) of low populations (i.e. <1.0 log CFU/g) of *E. coli* O157:H7. Hyeon et al. *(46)* developed a multiplex real-time PCR assay capable of detecting both *Salmonella* and *Cronobacter* in powdered infant formula at levels as low as 0.1 CFU/g after a 12-h enrichment in a 1:9 dilution of BPW. However, the same levels could not be detected following a shorter enrichment incubation of 9 h.

Osmotic shock occurs when desiccated pathogens in low-a_w foods are added to preenrichments; hence, increasing the osmolality of the enrichment by adding solutes may improve recovery of injured cells *(27)*. Cordier *(27)* summarized studies in which a "soak" method was used by sprinkling contaminated powders on the surface of the enrichment medium and allowing the matrix to slowly absorb the liquid over 60 min. This approach significantly improved the recovery of *Salmonella* in freeze-dried cells as well as in contaminated nonfat dried milk and dehydrated soy proteins. However, the method was not as conclusive for contaminated yeast powder.

In order to overcome competitive microflora in the preenrichment broth, malachite green has been added to inhibit non-*Salmonella* cells. In another study, the addition of brilliant green to media for the recovery of thermally injured salmonellae recovered a population of injured cells equivalent to the population of cells that were resuscitated on nonselective tryptic soy agar *(38)*.

In addition to newer molecular methods, immunological methods to improve detection of *Salmonella* have also been explored. Lepper et al. *(59)* compared the immunoconcentration *Salmonella*/VIDAS *Salmonella* (SLM) immunoassay method for detection of low levels of *Salmonella* with the BAM-AOAC method with ground pepper, nonfat dried milk, soy flour, milk chocolate, and dried whole egg. The percentages of agreement between the two methods for low contamination levels were 91% for milk chocolate, 99% for trial 3 of nonfat dried milk, 87% for black pepper, 83% for whole dried egg, and 64% for soy flour. There was no

statistical difference ($P > 0.05$) between the two methods when testing low levels of *Salmonella*.

PCR INHIBITORS

Rijpens and Herman *(69)* reported a number of inhibitors that could impede pathogen detection in PCR-based methods. These inhibitors included proteinases in milk, which can degrade *Taq* polymerase, calcium ions in milk, which can inhibit PCRs, and high $MgCl_2$ concentrations in irgasan ticarcillin chlorate medium, which can also inhibit PCRs. Other compounds that inhibit PCRs include certain phenolic groups *(56)* and hemes *(45)*. Goodridge et al. *(33)* reported the presence of compounds in foods that can inhibit DNA polymerase, bind magnesium, or denature the DNA. Some of these compounds are polyphenolic compounds, proteinases, complex polysaccharides, and fats.

In addition, when molecular techniques are used to detect pathogens in dry pet foods, DNA amplification reactions may be inhibited by the large numbers of background microorganisms *(29)*. For traditional methods, preenrichments, no shorter than 18 to 24 h, are recommended. Immunological or DNA hybridization–based methods have been approved for pathogen detection in dried pet food; however, extremely complex food matrices within these products have led to interference or inhibition in alternative testing methods *(29)*. Such interferences are not unique to pet foods. Polyphenols in chocolate can also inhibit PCRs. Hence, chocolate and other food products must be tested by methods other than PCR assays unless inhibitors can be neutralized.

Physical separation methods can be used in sample preparation to minimize the inhibiting effect of given food ingredients prior to a PCR assay *(71)*. These methods include the following.

1. Filtration. Sieving combined with filtration offers the best results when conducted in succession, and subsequent filtering using decreasing pore sizes further minimizes particulates.
2. Dielectrophoresis. The target bacteria move based on their charges; however, a minimum level of 7 log CFU is needed.
3. Sonication. Particles are moved in resonance nodes based on energy and wavelength.
4. Low-speed centrifugation. Particles are separated from suspended bacteria in a liquid medium at forces of $\leq 1{,}000 \times g$.
5. Density gradient centrifugation. Heterogeneous liquid food separates based on the density of the food ingredients.
6. Adsorption. Van Der Waal's forces, electrostatic interaction, hydrophobic interaction, and hydrogen bonding are utilized to mediate nonspecific adsorption to solid materials such as metal hydroxides, ion exchange resins, and lectins to separate food pathogens from food matrices into phases.

Tatavarthy et al. *(77)* tested three real-time PCR methods (Pacific Regional Laboratory Southwest [PRLSW], Applied Biosystems MicroSEQ, and GeneDisc platforms) for the detection of *Salmonella* in inoculated clove samples. The authors reported several false-negative results, which they surmised could be due to PCR inhibitors contained in cloves or its primary essential oil, eugenol. The authors concluded that the boil preparation DNA extraction method was the most efficient, regarding sensitivity and selectivity, and was superior to the MagMax and LyseNow methods for detecting *Salmonella* in cloves. The boil preparation matched or exceeded the standard DNA extraction methods for all the PCR platforms. The PRLSW platform was the most accurate in segregating true positive samples from true negative samples for the three real-time PCR platforms tested. The results of these studies clearly indicate that PCR sample preparation must be carefully considered, including the addition of compounds to neutralize inhibitors, filtration, appropriate DNA extraction, or the use of immunomagnetic separation beads.

Jasson et al. *(48)* tested the recovery of low populations of sublethally injured as well as healthy salmonellae in chocolate by two detection methods: (i) iQ-Check *Salmonella* II real-time PCR (Bio-Rad, Hercules, CA) and (ii) VIDAS Easy SLM (bioMérieux, Marcy l'Étoile, France), comparing them with the standard ISO 6579:2005 culture method (International Organization for Standardization, Geneva, Switzerland). Regardless of detection method, BPW did not support recovery of sublethally injured cells; however, BPW supplemented with 100 g/L milk powder successfully recovered injured cells with all three methods. Injured salmonellae in one of three chocolate samples was undetectable by the real-time PCR, which could be a function of poor enrichment or inhibition of PCR products. Cocoa contains a high concentration of polyphenols and polysaccharides (*Taq* polymerase inhibitors) inhibitory to PCRs *(37, 66)*. The authors concluded that a prolonged enrichment step may be required or an additional selective enrichment could be added prior to the PCR to improve sensitivity and avoid false-negative results with sublethally injured cells.

Some newer polymerases, i.e., *Tfl* and r*Tth* polymerases, are more recalcitrant to inhibitory substances and can be used in the place of *Taq* polymerase; they are especially useful when testing meat and cheese samples *(1)*. There are also some facilitators that can be added to the PCR to improve the polymerase activity in contact with inhibitors. These include bovine serum albumin, dimethyl sulfoxide, Tween 20, Triton-X, and betaine *(1, 56, 65, 68, 87)*.

SUMMARY AND CONCLUSIONS

There are numerous obstacles, such as antimicrobial compounds, particulates, PCR inhibitors, and fatty matrices, to the detection of foodborne pathogens in low-a_w foods. New approaches should be sought to increase the sensitivity of pathogen testing in low-a_w foods and to overcome the effects of various inhibitors and antimicrobials. The FDA and other laboratories are working toward this goal.

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