Evaluation of RAPID'*Staph*™ Medium for Enumeration of Coagulase-Positive *Staphylococcus aureus* in Selected Foods

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Introduction

Staphylococcus aureus is a gram-positive spherical bacterium that is seen microscopically in pairs, short chains, or clusters. Some strains are capable of producing a highly heat-stable toxin that can cause nausea, vomiting, and diarrhea in humans within 1–7 hr of ingestion (Bean et al. 1996). Foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation are frequently involved in staphylococcal food poisoning (Centers for Disease Control and Prevention 1997). *Staphylococcus aureus* is one of the most common etiological agents to cause foodborne outbreaks (Bean et al. 1996). Contributing factors leading to food contamination include improper holding temperatures, contaminated equipment, inadequate cooking, and poor personal hygiene (Bean et al. 1996). Humans are the primary reservoir for food contamination, as staphylococci can be found in nasal passages, in the throat, and on the skin of healthy individuals (Genigeorgis 1989). Staphylococci are also one of the predominant bacteria found on slaughtering and processing lines of poultry facilities, with many isolated strains possessing resistance to multiple antibiotics (Huys et al. 2005). Current culture methods for enumeration of *S. aureus* require an incubation time of 48 hr. The principle of RAPID'*Staph* medium relies on the capacity of *S. aureus* to reduce tellurite (leading to production of black colonies) and to provoke proteolysis of egg yolk (production of clear halo around each colony) (Figure 1). The medium includes a proprietary peptone mixture in addition to meat and yeast extracts; it provides nutrients to allow growth of the bacteria in 24 hr. Glycine and lithium chloride inhibit the growth of competing bacteria, adding to the selectivity of the medium, while sodium pyruvate stimulates the growth of *S. aureus*, increasing sensitivity.

This report summarizes an Association of Official Analytical Chemists (AOAC) International Research Institute validation study. In this study, RAPID'*Staph* medium was granted Performance Tested method status by AOAC-RI, certificate #080602.

Fig. 1. RAPID'*Staph* agar plate.

Methods

Inclusivity Study

The purpose of the inclusivity study was to ensure that all strains of *S. aureus* tested could be positively identified on RAPID'*Staph* plates. Thirty food and clinical isolates of *S. aureus* were selected. Strains were cultured at 37°C for 24 hr in brain heart infusion (BHI) broth (Bio-Rad Laboratories). Cultures were plated on RAPID'*Staph* agar and incubated at 37°C for 24 hr. Typical colony morphology (black colony with clear halo) was recorded.

Exclusivity Study

The purpose of the exclusivity study was to determine the ability of RAPID'*Staph* medium to discriminate target organisms from nontarget organisms. Forty non-*S. aureus* organisms were selected (Table 1). Strains were cultured at 37°C for 24 hr in BHI broth (EMD Chemicals). Cultures were plated on RAPID'*Staph* agar and incubated at 37°C for 24 hr. Typical colony morphology (black colony with clear halo) was recorded.

Table 1. Exclusivity study results.

Strain	Strain ID*	Source**	RAPID'*Staph* Result
Alcaligenes faecalis	ATCC 8750	Unknown	No growth
Bacillus cereus	ATCC 11778	Unknown	No growth
Candida albicans	ATCC 10231	Human	No growth
Carnobacterium spp.	Ad503M10	Raw milk	No growth
Citrobacter braaki	ATCC 51113	Snake	No growth
Citrobacter freundii	ATCC 8090	Unknown	No growth
Edwardsiella tarda	ATCC 15947	Human feces	No growth
Enterobacter cloacae	ATCC 13047	Spinal fluid	No growth
Escherichia blattae	ATCC 29907	Insect	No growth
Escherichia coli	ATCC 25922	Clinical	No growth
Escherichia coli O157:H7	ATCC 35150	Human feces	No growth
Escherichia coli O157:H7	ATCC 43895	Raw hamburger meat	No growth
Escherichia ferguson	ATCC 11698	Infant diarrhea	No growth
Escherichia vulneris	ATCC 29943	Wound	No growth
Hafnia alvei	ATCC 29926	Human	No growth
Klebsiella pneumonia	ATCC 13883	Unknown	No growth
Kluyveromyces lactis	Ad004	Salad	No growth
Kluyveromyces marxianus	AdCLIB720	Raw milk	No growth
Kocuria rosea	CIP71.15	Tap water	No growth
Lactobacillus hilgardii	ATCC 8290	Wine	No growth
Listeria ivanovii	CIP108466	Clinical	Atypical colonies
Listeria monocytogenes	ATCC 19115	Human	Atypical colonies
Macrococcus caseolyticus	CIP100755	Milk	No growth
Microbacterium spp.	BAA-799	Fresh water	No growth
Micrococcus luteus	ATCC 10240	Air	No growth
Morganella morganii	ATCC 35200	Unknown	No growth
Pseudomonas aeruginosa	ATCC 27853	Blood culture	No growth
Rhodococcus equi	Ad010	Soil	No growth
Salmonella infantis	ATCC 51741	Pasta	No growth
Shigella sonnei	RTU8	Human feces	No growth
Staphylococcus carnosus	Ad011	Sausage	No growth
Staphylococcus carnosus	Ad012	Sausage	No growth
Staphylococcus cohnii	Ad156	Chicken skin	No growth
Staphylococcus epidermidis	Ad150	Minced beef	No growth
Staphylococcus epidermidis	ATCC 12228	Unknown	No growth
Staphylococcus equorum	CIP103502	Horse skin	No growth
Staphylococcus hyicus	CIP81.58	Pork	Atypical colonies
Staphlyococcus saprophyticus	ATCC 15305	Urine	No growth
Staphylococcus xylosus	Ad151	Minced beef	No growth
Streptococcus pyogenes	ATCC 19615	Human pharynx	No growth

* Ad and Collection de l'Institut Pasteur (CIP) strain identification numbers assigned by Adria Developpement Lab, Quimper, France. RT strain identification number assigned by rtech laboratories, Arden Hills, MN, USA.

** Unknown = No American Type Culture Collection (ATCC) record of source information.

Method Comparison Study

Inoculation and Sample Preparation — Inoculation cultures were selected from the culture collection of rtech laboratories. A different *S. aureus* strain was inoculated into samples of each food. A sample of frozen stock culture was streaked onto trypticase soy agar (TSA) with 5% sheep blood (Becton Dickinson) to isolate colonies. Culture purity was examined to ensure that only one colony morphology was present on the plate. A single isolated colony was selected and subcultured into BHI broth (EMD Chemicals) and incubated at 37°C for 24 hr. Serial dilutions were made in Butterfield's phosphate buffer (0.3 mM, pH 7.2) and plated to TSA (EMD Chemicals) for counting. The culture was stored at 4°C until the titer was verified.

Pasteurized whole milk, custard pie, processed ham, and smoked salmon were purchased from a grocery store. Three inoculation levels of *S. aureus* were targeted: 10–100, 100–1,000, and 1,000–10,000 *S. aureus*/g. A 300 g batch of food for each inoculation level was divided into four quadrants

and artificially seeded with the target organism. Each quadrant was hand mixed for 2 min. Then the quadrants were combined and hand mixed for 5 min. Following inoculation, test products were held at 4°C for 48 hr to stabilize before analysis. Five 50 g subsamples of each food batch were randomly assigned numbers and blind coded. Each 50 g sample was homogenized by blending with 450 ml of Butterfield's phosphate buffer for 2 min. Five uninoculated control samples were processed in the same manner. One sample homogenate was prepared for analysis by both the reference method and the RAPID'*Staph* method. Processed ham was tested in two different studies, an internal method comparison study and an independent laboratory verification study.

AOAC Official Methods 975.55 and 987.09 — Reference method samples were processed according to AOAC Official Method 975.55 (AAOAC International 2000a). Specifically, 1 ml of each homogenized sample, or a dilution thereof, was divided (0.4 ml, 0.3 ml, 0.3 ml) among three Baird-Parker agar plates (EMD Chemicals) and spread using a sterile, bent glass streaking rod. Plates were inverted and incubated at 37°C for 45–48 hr. Plates containing 20–200 colonies were selected for confirmation according to AOAC Official Method 987.09 (AAOAC International 2000b). Five typical colonies were selected from each sample and transferred into 0.2 ml of BHI, streaked onto TSA slants (Becton Dickinson), and incubated at 35°C for 18–24 hr. Reconstituted coagulase plasma (500 µl) with EDTA (Becton Dickinson) was added to BHI cultures, mixed thoroughly, and incubated at 37°C. Cultures were examined periodically over a 6 hr period for clot formation. Any degree of clot formation was considered a positive coagulase reaction. Counts from triplicate plates with positive coagulase reactions were added, multiplied by the percentage of confirmed colonies, and then multiplied by the appropriate dilution factor. This number was recorded as *S. aureus*/g of food.

RAPID'*Staph* Method — For samples analyzed according to the RAPID'*Staph* method, serial dilutions, determined by target inoculation level, were made in Butterfield's phosphate buffer. Two plating methods were tested. For the first method, 1 ml of each sample was removed and inoculated onto three dried RAPID'*Staph* plates (0.4 ml, 0.3 ml, 0.3 ml) by the spread plate technique. In the second method, used for the mid- and high-level samples, which had an estimated contamination of >150 *S. aureus*/g, 0.1 ml of each sample was inoculated onto one dried RAPID'*Staph* plate by the spread plate technique. Overall, plating methods tested by inoculation level can be summarized as follows: uninoculated controls, 1 ml over three plates; low level, 1 ml over three plates; mid level, 1 ml over three plates and 0.1 ml on one plate; high level, 1 ml over three plates and 0.1 ml on one plate. All plates were incubated at 37°C for 24 ± 2 hr. Black colonies with clear halos were counted as suspect *S. aureus*. Five suspect colonies were selected from each sample and confirmed according to AOAC Official Method 987.09. Counts from RAPID'*Staph* plates with positive coagulase reactions were added, multiplied by the percentage of confirmed colonies, and then multiplied by the appropriate dilution factor. This number was recorded as *S. aureus*/g of food.

Statistical Analysis — Statistical analysis was performed according to guidelines of AOAC International (Feldsine et al. 2002). The mean result for each level and method was calculated. A one-way analysis of variance (paired Student's *t*-test) was used to determine whether there was a significant difference ($p < 0.05$) between the AOAC Official Method and the RAPID'*Staph* method. Repeatability (Sr), or measurement of precision, is the closeness of agreement between two mutually independent test results. This was calculated for each inoculation level of each food and stated in terms of standard deviation. Relative standard deviation to the mean (RSDr) was also calculated. Accuracy, or method agreement, was used to determine the closeness of agreement between the reference method and the test method.

Results

In pasteurized whole milk samples, no statistical difference ($p > 0.05$) between the two methods using the 1 ml inoculum (Table 2) was observed; however, the high contamination level for this food with the 0.1 ml inoculum was statistically significant ($p < 0.05$). The counts for the 0.1 ml RAPID'*Staph* method were higher than those for the AOAC method. Data from custard pie samples yielded no statistical difference between the RAPID'*Staph* and the AOAC Official Method. The results for the internal study on processed ham showed a statistical difference ($p < 0.05$) between the RAPID'*Staph* and AOAC methods at the low inoculation level using the 1 ml inoculum. The RAPID'*Staph* method counts were more repeatable. The AOAC method had one replicate count of 180 *S. aureus*/g, while the other replicates were 80, 70, 70, and 70 *S. aureus*/g. This result could have been produced by a plating error. If this one sample (and the corresponding RAPID'*Staph* replicate) were removed, there would be no statistical difference between the two methods ($p > 0.05$). The independent laboratory verification of processed ham contamination levels showed no statistical difference between the two methods. Results from smoked salmon samples were similar to those for pasteurized whole milk. No statistical difference ($p > 0.05$) between the two methods using the 1 ml inoculation scheme was seen; however, the high level of contamination in the 0.1 ml inoculation method represented a statistical difference ($p < 0.05$). The counts for the 0.1 ml RAPID'*Staph* method were higher than those for the AOAC method.

Table 2. Analysis of variance. A p-value of <0.05 indicates a statistical difference.

Food	Inoculation Scheme	Level	p-Value
Pasteurized whole milk	1 ml	Low	0.39
		Mid	0.24
		High	0.05
	0.1 ml	Mid	0.15
		High	0.003
Custard pie	1 ml	Low	0.07
		Mid	0.11
		High	0.39
	0.1 ml	Mid	0.18
		High	0.26
Processed ham (internal study)	1 ml	Low	0.03
		Mid	0.10
		High	0.94
	0.1 ml	Mid	0.60
		High	0.25
Processed ham (independent study)	1 ml	Low	0.96
		Mid	0.28
		High	0.55
	0.1 ml	Mid	0.49
		High	0.68
Smoked salmon	1 ml	Low	0.77
		Mid	0.06
		High	0.51
	0.1 ml	Mid	0.05
		High	0.03

Discussion

Two RAPID'*Staph* methods were validated in this study. The first method required removing 1 ml of sample and plating it on three RAPID'*Staph* agar plates. This method was tested to evaluate whether samples could be processed according to the reference method protocol but substituting Baird-Parker agar with RAPID'*Staph* agar in order to obtain results 24 hr earlier. The second method validated involved removing 0.1 ml of sample homogenate and plating it to one RAPID'*Staph* plate. This is a cost-saving method, as one plate is required instead of three. This method, however, can only be used if *S. aureus* contamination is expected to be >150 cfu/ml. If the contamination level is <150 *S. aureus*/g, removing 0.1 ml will dilute the sample 10-fold and cause the number of colonies on the RAPID'*Staph* plate to fall below the statistically accurate counting range of the plate (15–150 colonies). Therefore, this method was only tested at the mid- and high-inoculum ranges. To reflect accurate counts, it is important to perform calculations with the appropriate dilution factor, no matter which plating method is used.

Although the method comparison studies demonstrated excellent correlation between the RAPID'*Staph* method and AOAC Official Method 975.55, statistical differences were observed for milk and smoked salmon at the high inoculation level using the 0.1 ml inoculation method. After the counts on the plates were verified and the coagulase tests were confirmed, it was concluded that the RAPID'*Staph* plate counts for this level were both more repeatable and higher than the AOAC Official Method counts. It is much easier to plate 0.1 ml of liquid on an agar plate than it is to plate 0.3 ml or 0.4 ml. When plating 0.3 ml or 0.4 ml, the liquid saturates the plate and cannot be completely absorbed, especially if the plate is slightly wet. Some culture could run off to the sides of the plate, resulting in a lower count. Some plates may be drier than others when plating occurs, so this is probably a random event; the results seen with the custard pie and ham matrices showed no statistical differences with this inoculum method. The method specifies that the plates be dried before use. A wet plate is a deviation from the method.

Conclusions

RAPID'*Staph* agar allows enumeration and differentiation of coagulase-positive *S. aureus* in 24 hr. When compared with AOAC Official Method 975.55, RAPID'*Staph* agar was shown to be an effective and efficient alternative medium for enumeration of *S. aureus* in pasteurized whole milk, custard pie, processed ham, and smoked salmon. For convenience, prepared media were used for this validation study; however, in additional ruggedness studies (data not shown), no differences were observed between this medium formulation and dehydrated media.

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