



**U.S. FOOD & DRUG
ADMINISTRATION**

Bacteriological Analytical Manual
Chapter 12: *Staphylococcus aureus*
March 2016 Edition

Table of Contents

AUTHORS	3
REVISION HISTORY	3
INTRODUCTION	3
DIRECT PLATE COUNT METHOD	4
MOST PROBABLE NUMBER METHOD FOR <i>STAPHYLOCOCCUS</i> SPP.	7
REFERENCES	7

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Revision History

- March 2016: The temperature for Incubation of *S. aureus* was changed to 35-37°C, from 35°C.

Introduction

Staphylococcus aureus is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation. *S. aureus* can cause severe food poisoning. It has been identified as the causative agent in many food poisoning outbreaks and is probably responsible for even more cases in individuals and family groups than the records show. Foods are examined for the presence of *S. aureus* and/or its enterotoxins to confirm that *S. aureus* is the causative agent of foodborne illness, to determine whether a food is a potential source of "staph" food poisoning, and to demonstrate post-processing contamination, which is generally due to human contact or contaminated food-contact surfaces. Conclusions regarding the significance of *S. aureus* in foods should be made with circumspection. The presence of a large number of *S. aureus* organisms in a food may indicate poor handling or sanitation; however, it is not sufficient evidence to incriminate a food as the cause of food poisoning. The isolated *S. aureus* must be shown to produce enterotoxins. Conversely, small staphylococcal populations at the time of testing may be remnants of large populations that produced enterotoxins in sufficient quantity to cause food poisoning. Therefore, the analyst should consider all possibilities when analyzing a food for *S. aureus*.

Methods used to detect and enumerate *S. aureus* depend on the reasons for testing the food and on the past history of the test material. Processed foods may contain relatively small numbers of debilitated viable cells, whose presence must be demonstrated by appropriate means. Analysis of food for *S. aureus* may lead to legal action against the party or parties responsible for a contaminated food. The methods of analysis for *S. aureus* that have been studied collaboratively and found suitable for use in providing the type of information necessary for FDA requirements are presented in this chapter.

There has been considerable controversy about the significance and correct method of reading the coagulase test. Research results have indicated that the weak coagulase activity represented by 1+, 2+, and 3+ reactions seldom corresponds with other criteria associated with *S. aureus* (4). A consensus of peers has established that a 4+ coagulase reaction is necessary for unquestioned identification of *S. aureus*. Those strains suspected of being *S. aureus* on the basis of coagulase reactions of less than 4+ should be confirmed by other tests, such as anaerobic glucose fermentation, lysostaphin sensitivity, and thermonuclease production. Studies of colonial morphology on Baird-Parker agar, lysostaphin sensitivity, coagulase and thermonuclease production, and glucose and mannitol fermentation

were conducted on 100 enterotoxigenic and 51 nonenterotoxigenic strains of *S. aureus* (3). In all cases, the reactions of enterotoxigenic and nonenterotoxigenic strains varied by 12% or less. This research indicates that none of these tests can be relied upon to differentiate toxic and nontoxic staphylococci.

Direct Plate Count Method

This method is suitable for the analysis of foods in which more than 100 *S. aureus* cells/g may be expected. It conforms to the method in ref. 1.

A. Equipment and materials

1. Same basic equipment as for conventional plate count (Chapter 3).
2. Drying cabinet or incubator for drying surface of agar plates
3. Sterile bent glass streaking rods, hockey stick or hoe-shaped, with fire-polished ends, 3-4 mm diameter, 15-20 cm long, with an angled spreading surface 45-55 mm long

B. [Media](#) and [reagents](#)

1. Baird-Parker medium ([M17](#))
2. Trypticase (tryptic) soy agar (TSA) ([M152](#))
3. Brain heart infusion (BHI) broth ([M24](#))
4. Coagulase plasma (rabbit) with EDTA
5. Tolidine blue-DNA agar ([M148](#))
6. Lysostaphin (Schwartz-Mann, Mountain View Ave., Orangeburg, NY 10962)
7. Tryptone yeast extract agar ([M165](#))
8. Paraffin oil, sterile
9. 0.02 M phosphate-saline buffer ([R61](#)), containing 1% NaCl
10. Catalase test ([R12](#))

C. Preparation of sample (see BAM [Chapter 1](#)).

D. Isolation and enumeration of *S. aureus*

1. For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum

is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35-37°C. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasionally from various foods and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

2. Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear at higher dilutions, use these plates for the enumeration of *S. aureus*, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

E. Coagulase test

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml BHI broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of BHI suspension. Incubate BHI culture suspension and slants 18-24 h at 35-37°C. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the BHI culture and mix thoroughly. Incubate at 35-37°C and examine periodically over 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (4). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically. A latex agglutination test (AUREUS TEST™, Trisum Corp., Taipei, Taiwan) may be substituted for the coagulase test if a more rapid procedure is desired.

F. Ancillary tests

1. Catalase test. Use growth from TSA slant for catalase test on glass slide or spot plate, and illuminate properly to observe production of gas bubbles.
2. Anaerobic utilization of glucose. Inoculate tube of carbohydrate fermentation medium containing glucose (0.5%). Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 35-37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube,

indicating presence of *S. aureus*. Run controls simultaneously (positive and negative cultures and medium controls).

3. Anaerobic utilization of mannitol. Repeat 2, above, using mannitol as carbohydrate in medium. *S. aureus* is usually positive but some strains are negative. Run controls simultaneously.
 4. Lysostaphin sensitivity. Transfer isolated colony from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsify. Transfer half of suspended cells to another tube (13 × 100 mm) and mix with 0.1 ml phosphate-saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 0.02 M phosphate-saline buffer containing 1% NaCl) to original tube for concentration of 25 µg lysostaphin/ml. Incubate both tubes at 35-37°C for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *S. aureus* is generally positive.
 5. Thermostable nuclease production. This test is claimed to be as specific as the coagulase test but less subjective, because it involves a color change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35-37°C. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.
- G. Some typical characteristics of 2 species of staphylococci and the micrococci, which may be helpful in their identification, are shown in Table 1.

Table 1. Typical characteristics of *S. aureus*, *S. epidermidis*, and micrococci^(a)

Characteristic	<i>S. aureus</i>	<i>S. epidermidis</i>	Micrococci
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
Anaerobic utilization of glucose	+	+	-
mannitol	+	-	-

^a +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.

Most Probable Number Method for *Staphylococcus* spp.

The most probable number (MPN) method (2) is recommended for routine surveillance of products in which small numbers of *S. aureus* are expected and in foods expected to contain a large population of competing species.

- A. Equipment and materials--Same as for Direct Plate Count Method, above.
- B. Media and reagents--Same as for Direct Plate Count Method, above. In addition: Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate (M154a).
- C. Preparation of sample--Same as for Direct Plate Count Method, above.
- D. Determination of MPN

Inoculate 3 tubes of TSB containing 10% NaCl and 1% sodium pyruvate (B, above) with 1 ml portions of decimal dilutions of each sample. Highest dilution must give negative endpoint. Incubate tubes 48 ± 2 h at 35-37°C. Using 3 mm loop, transfer 1 loopful from each tube showing growth (turbidity) to plate of Baird-Parker medium with properly dried surface. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate plates 48 h at 35-37°C. From each plate showing growth, transfer at least 1 colony suspected to be *S. aureus* to BHI broth (see D and E of Direct Plate Count Method, above). Continue procedure for identification and confirmation of *S. aureus* (E and F, Direct Plate Count, above). Report *S. aureus*/g as MPN/g, according to tables in [Appendix 2, MPN Determination](#).

References

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Original Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Chapter 12.