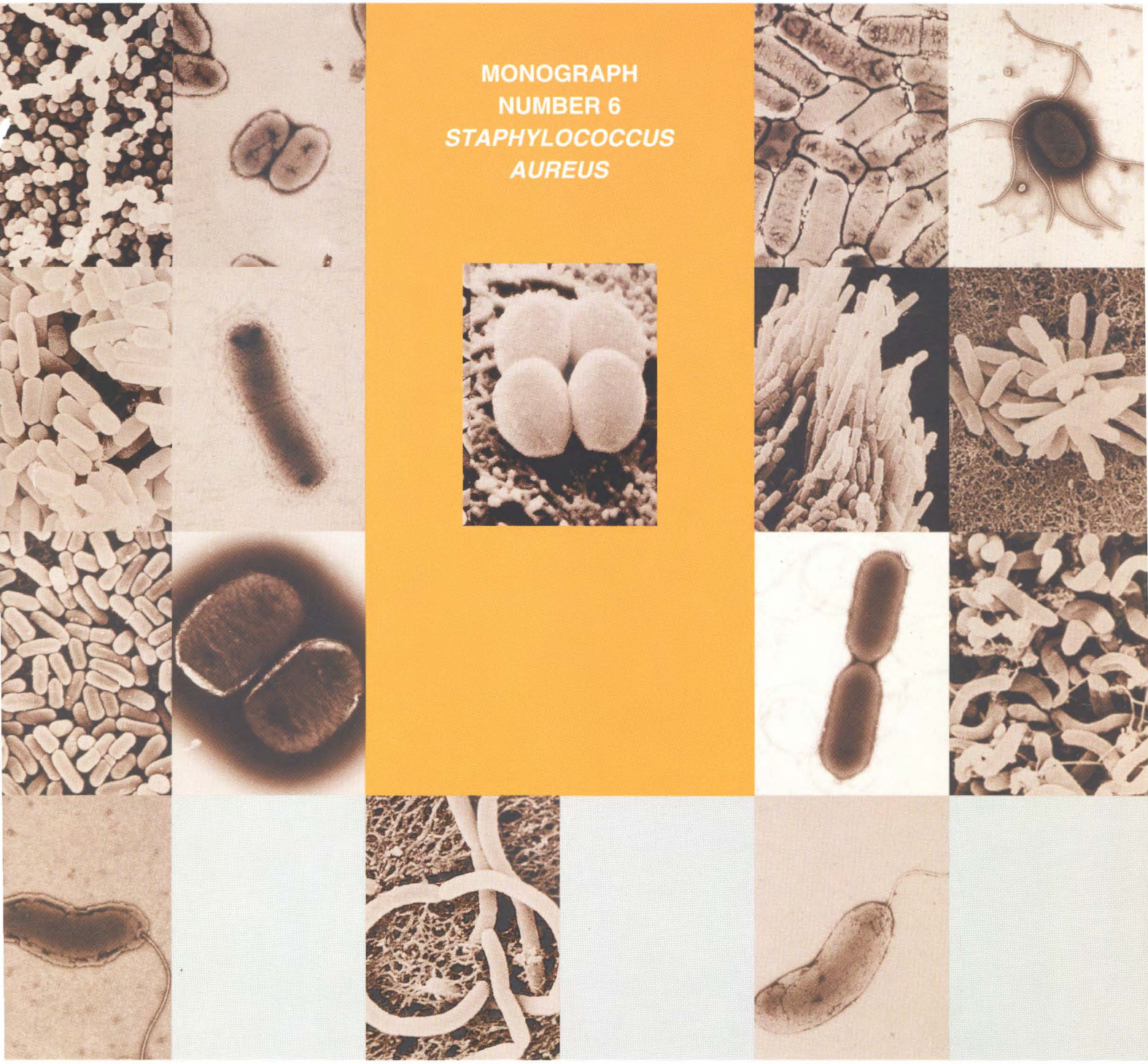
Food-borne Pathogens



OXOID

SETTING STANDARDS

FOOD-BORNE PATHOGENS MONOGRAPH NUMBER 6 *STAPHYLOCOCCUS AUREUS*

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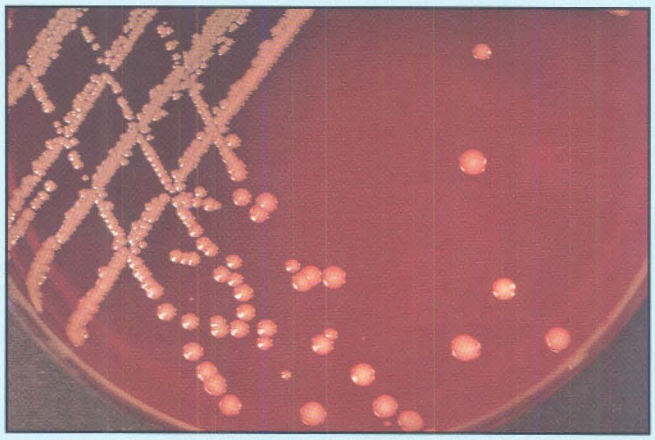
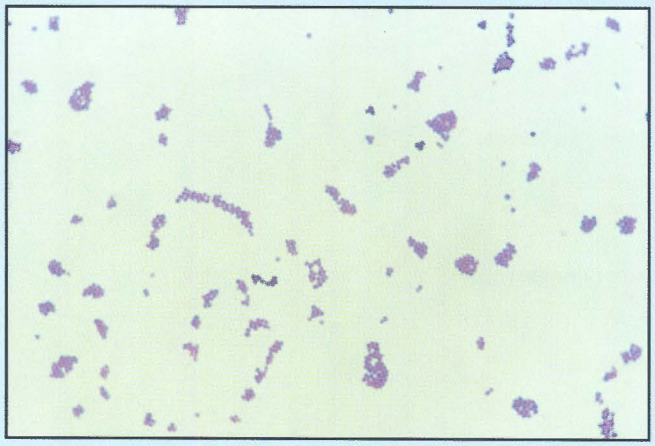
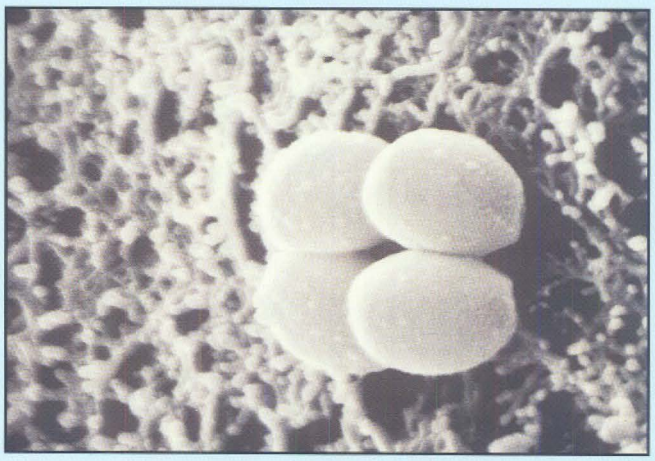


Typical appearance of *Staphylococcus aureus* colonies on blood agar.



Gram-stain appearance of *Staph. aureus* in culture. Note the characteristic arrangement of cells in "clusters of grapes".
Oxoid Marketing collection.



Electron microscopy appearance of a cluster of *Staph. aureus* cells.
Oxoid Marketing collection.

Staphylococcus aureus

Introduction

The principal interest of food microbiologists in staphylococci lies in the capacity of a sizeable proportion of strains of the species *Staphylococcus aureus* to produce enterotoxin which induces food poisoning characterised by nausea, vomiting, retching and stomach cramps, often accompanied by diarrhoea. Generally patients recover rapidly from the illness, usually in one to two days. Although the symptoms are very unpleasant, staphylococcal food poisoning is seldom dangerous although dehydration may be sufficiently great to require intravenous infusion.

Some other *Staphylococcus* species have also been shown to produce enterotoxin.

Most domestic animals harbour staphylococci and *Staph. aureus* is a common cause of bovine mastitis. If milk from infected cattle is used for cheese making, then food poisoning is likely to occur after eating the cheese. In humans, *Staph. aureus* inhabits the nose and may readily be transferred to food during preparation and handling. If the organism is able to multiply sufficiently enterotoxin may be produced.

Staphylococcal food poisoning is a frequent cause of food-borne illness and has probably occurred since antiquity. The first outbreak to be recorded was in 1884 when several hundred cases were attributed to eating Cheddar cheese.[1] It was not until 1914 however, that staphylococci were clearly identified as food poisoning organisms when illness occurred amongst persons who had consumed milk from a cow with mastitis.[2] The significance of this event was slow to be recognised but in 1930 further evidence was provided when a pigmented Gram-positive coccus was isolated from a cake involved in a food-poisoning incident.[3] The link was finally established when the typical symptoms of food poisoning were seen in human volunteers who had swallowed a broth culture of the organism.[4]

Food poisoning by staphylococci is probably considerably under-reported because of its short duration. The greatest number of cases typically occur in summer when improperly stored food is exposed to the warmest temperatures. Staphylococcal food poisoning can be prevented by measures directed towards avoiding contamination during food preparation and handling, and by refrigerating high-risk foods until shortly before they are eaten.

Staph. aureus produces a great variety of exotoxins, enzymes and other factors associated with infectivity and disease. The commonest manifestation of *Staph. aureus* infection is familiar because of the superficial skin infections (boils) the organism causes. *Staph. aureus* is also capable of producing far more serious, deep-seated, infections including abscesses, osteomyelitis and septicaemia.

The surgeon Alexander Ogston demonstrated that pus containing organisms having a characteristic microscopic appearance, when injected into mice caused a disease similar to that seen in man.[5] He showed that disease failed to occur following inoculation of pus that had been heated or exposed to phenol.

In the same year Pasteur was able to culture the organisms from furuncle pus inoculated into a broth culture medium.[6] Later, Ogston named the organism *Staphylococcus* from its appearance which resembles bunches of grapes.[7] Microscopically, staphylococci appear as Gram-positive spherical to oval-shaped cells. Staphylococci are catalase-positive (a useful distinguishing feature) and grow well on ordinary nutrient media under aerobic and anaerobic conditions. Growth occurs over a temperature range of 7 to 48°C with optimal growth in the region of 35 to 37°C. *Staph. aureus* is not exceptionally heat resistant. An important consideration in food microbiology is the relative tolerance shown by this organism towards salt and low water activity. Fortunately the extremes of conditions under which *Staph. aureus* will produce toxin are less broad than those the organism will tolerate.

In addition to carriage in the nose, *Staph. aureus* is also present on the skin of normal individuals. A variety of mammals carry staphylococci which inhabit the same body sites. Different species tend to be associated with particular animals. Some of these species are closely similar to *Staph. aureus* and this can cause confusion in routine identification procedures. Some are able to produce enterotoxin.

Because of the constant shedding of staphylococci by carriers, the implications for hygienic handling of food are considerable. A high level of control of the numbers of *Staphylococcus* cells present in food and the way the food is handled after preparation is essential.

The principal means of detection of staphylococci is by isolation on selective culture media which are also designed to demonstrate a number of characteristics useful in presumptive identification. The usual (and often the only) confirmatory test applied to colonies presumed to be *Staph. aureus* is the coagulase test. This test, which indicates expression of the exoprotein coagulase, was thought to be specific for *Staph. aureus* but now it is known that other species of staphylococci are also coagulase-positive. A positive coagulase test is indicated by coagulation of blood plasma resulting in the formation of a clot. When results of coagulase testing are equivocal it is essential that other tests are performed.

This publication reviews detection and identification methods which depend on culture of the organism. Coagulase and tests for coagulase are considered in detail. Differentiation of the various species important in food microbiology will be described. Enterotoxin and methods used in its detection are discussed.

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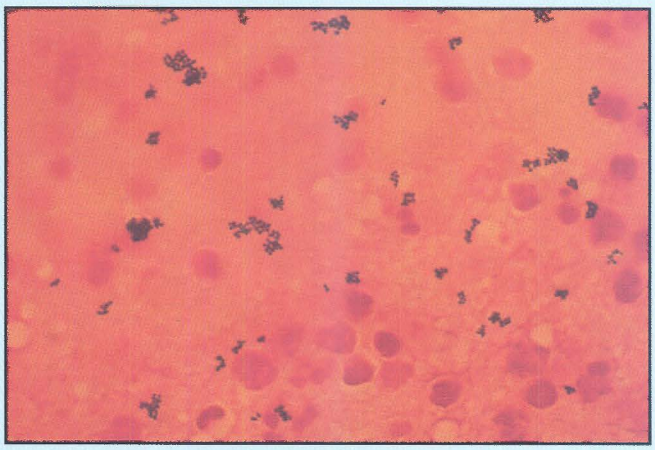

Gram stain of pus showing cells of *Staph. aureus.*

Dr Matthew Dryden, Royal Hampshire County Hospital, Winchester.

The occurrence of *Staphylococcus aureus* in foods

Staphylococcal food poisoning is a common cause of gastro-enteritis throughout the world. It is therefore of vital concern to the food industry.

Because the various species of staphylococci are associated with particular animals, a number of species may be contributed to the processing environment by the different animal products passing through. Species of staphylococci associated with animals are shown in Table 9. In the majority of these associations the animal named appears not to be the natural host.

A number of species, including some that do not produce coagulase, are able to produce enterotoxins. However, *Staphylococcus aureus* has an exceptional capacity for causing food poisoning and detection methods for staphylococci are strongly directed towards this species.

Staph. aureus is commonly carried in the nose and on the skin of humans and therefore persons involved in food manufacture and preparation may contribute to the overall population of staphylococci in foods.

Unlike other pathogens found in food, the presence of *Staph. aureus* does not necessarily constitute a hazard since the safety of the food is conditional on the absence of enterotoxin and not that of the organism. However, the presence of *Staphylococcus* in foods that have been processed, and therefore should be free of the organism, is a valuable indicator of unsatisfactory post-process hygienic conditions and storage temperature.

Staph. aureus grows well under the usual conditions of pH and incubation temperature. It is not heat resistant although this can vary with strain and the growth phase it is in when subjected to heat. It is, however, exceptional in its relatively high tolerance of salt and reduced water activity and this obviously has implications for the safety of certain types of food. However, the extremes of the conditions that permit toxin to be produced are generally less than those that allow growth to occur.

The presence of small amounts of *Staph. aureus* on unprocessed foods is quite common, although as stated earlier, presence in processed foods may be much more significant. It is a frequent component of the skin flora of food animals and therefore can be expected to be present on raw poultry and other meats. The organism is a common inhabitant of the defeathering equipment in chicken processing plants and this contributes to spread amongst slaughtered birds.

Staph. aureus can be isolated from raw milk and numbers can be high if milk is taken from cows sick with mastitis. If the organisms survive pasteurisation, or if there is post-pasteurisation contamination of milk, *Staph. aureus* may subsequently be found in a variety of milk products although it is only some hard cheeses that have been associated with food poisoning.

Dried powders in infant formulae are a potential source of health problems for very young children. Concentrated liquid milk appears to hold little risk.

Because of the tolerance of *Staph. aureus* to high salt concentration and low water activity (aw) it can cause difficulties in the manufacture of salami and other fermented meats. It is also suspected to be the cause of food poisoning that has occurred following consumption of pickled vegetables. *Staph. aureus* grows well on a variety of vegetables and there have been reports of food poisoning resulting from eating cooked vegetables that had been kept warm for some time before serving.

Liquid and hard-boiled eggs, and pasta in which egg is a constituent, have caused food poisoning. Baked confectionery products containing cream classically appear to be associated with food poisoning although cream itself is not a good milieu for growth or toxin production.

Canned goods are generally not a problem but food poisoning has been caused by ingress of staphylococci into imperfectly sealed cans of mushrooms and corned beef.

Because *Staph. aureus* is ubiquitous and commonly present on food handlers, the variety of foods at risk is considerable. Problems tend to occur with cold dishes that require much handling in the preparation and presentation of buffet meals. It might be generalised that the more visually attractive a cold meal, the greater the risk of staphylococcal food poisoning.

Table 1 – Official Bodies that specify Detection Procedures and the Culture Media to be used.

Body	Enrichment	Plating	Other Media
American Public Health Association	Tryptone Soya broth + 10% or 20% sodium chloride and sodium pyruvate	Baird-Parker or Baird-Parker plasma-fibrinogen agar	Toluidine blue-DNA agar Brain-heart infusion
Australian standard AS1766.2.4 1994	Giolitti-Cantoni broth	Baird-Parker	
Canada: Health Protection Branch MFHPB – 21 1985		Baird-Parker	Brain-heart infusion Tryptone soya agar Blood agar, Nutrient agar Toluidine-blue-DNA agar Phenol red-Carbohydrate broth
France: AFNOR V08–057–1		Baird-Parker	Brain-heart infusion
Italy: Istituto Superiore di Sanita Rapporti ISTISAN 96/35		Baird-Parker	Brain-heart infusion
ISO/European/British Standard 4285 and 5763	Giolitti-Cantoni broth	Baird-Parker	Brain-heart infusion
Nordic Committee on Food Analysis, Number 66 2nd edition 1992		Baird-Parker	Blood agar

Selective enrichment for *Staphylococcus aureus*

Staphylococcus aureus may be present in small numbers in many foods and, in most, small numbers have little significance. Consequently enrichment culture generally is not as important in detection procedures as it is for many food pathogens and plating of samples directly onto a productive medium is usually sufficient.

The presence of *Staph. aureus* in foods must be considered in relation to the amount and types of accompanying flora; staphylococci compete poorly with indigenous food-borne bacteria: they are inhibited by antagonistic activities of other organisms, less successful in competition for nutrients and show greater susceptibility to changes in the food environment. Indeed, it can be conjectured that improvements in food processing plant design and hygiene might actually increase the risk of staphylococcal food poisoning by reducing the numbers of competing organisms present in the manufacturing environment enabling staphylococci to multiply more readily. Large numbers of cells are required for production of sufficient toxin to cause food poisoning. For foods that are terminally heat-processed, tests for viable *Staph. aureus* should be carried out before exposure to heat. In situations where growth may have occurred in a food, but the majority of cells may no longer be viable, a test for enterotoxin directly on the food may be more appropriate than enrichment of the few remaining cells. Alternatively, tests for the presence of high numbers of dead *Staph. aureus* are advisable. The presence of heat-stable thermonuclease produced by the organisms during growth may be a useful indicator of this.

The limited need for enrichment culture of *Staph. aureus* is probably responsible for the small number of media devised for the purpose, compared with the situation for some other food-borne pathogens. Selective media are used in Most Probable Number (MPN) techniques for detection of small numbers of *Staph. aureus* and, when necessary, for selective enrichment before subculture to agar media. A major requirement of enrichment media for any organism is that they must be capable of resuscitating sub-lethally damaged cells which are present in many foods which have been subjected to relatively mild preservation processes. Failure of this to occur may result in selective agents inhibiting the organism being sought and consequent failure to detect its presence.

Early media formulations took advantage of the tolerance shown by *Staph. aureus* to concentrations of sodium chloride that inhibit accompanying organisms. Tryptone soya broth with added salt has been used for many years.[1,2] Brain-Heart infusion broth with added salt has also been used.[3]

However, it has become apparent that concentrations of sodium chloride greater than 40 grams/litre are inhibitory to injured cells. The productivity of high salt content tryptone soya broth has been improved by incorporation of sodium pyruvate.[4] Nutritionally, tryptone soya broth is a desirable medium for growth of staphylococci and modifications to methodology designed to ensure a period for resuscitation to occur before exposure to salt have permitted the retention of selective tryptone soya broth in current procedures.[5]

A broth variant of mannitol-salt agar has been developed for selective enrichment of *Staph. aureus* in foods. Like the agar, this medium uses fermentation of mannitol for presumptive identification of the organism. However, the medium is likely not to detect stressed organisms because of the lack of both resuscitant in the formula and time for cell repair in the recommended procedure for its use.

Salt meat broth, which is cooked-meat medium containing 10% w/v of sodium chloride,[6] has also been recommended for selective isolation of staphylococci from foods and from faeces in investigations of food poisoning.[7] However, it too is likely to perform poorly when the *Staphylococcus* cells are stressed.

The use of sodium chloride for selection of *Staph. aureus* was derived from clinical bacteriology practice where numbers of *Staph. aureus* in pathological specimens is high compared with numbers in food samples. Giolitti and Cantoni[8] devised a medium more appropriate to food microbiology using potassium tellurite and glycine as selective agents active against Gram-negative and Gram-positive bacilli. Inhibition of micrococci is achieved by incubating tubes of medium under anaerobic conditions created by pouring sterile oil or paraffin wax onto the medium following inoculation. The long column of medium in a tube further assists formation of the low Eh conditions that are necessary. The presence of *Staph. aureus* is presumed if the medium blackens due to reduction of the potassium tellurite.

Giolitti-Cantoni medium is used in presence/absence testing and in an MPN technique for detection of very small numbers of *Staph. aureus* in dried milk and infant weaning foods in which multiplication of staphylococci can occur rapidly following reconstitution. The medium may also be used for the examination of meat and meat products but for this purpose it is necessary to reduce the content of potassium tellurite.[9]

Baird-Parker agar has become the most widely used selective agar in food microbiology (see Table 3) because of its superior productivity. It is not surprising therefore, that the successful features of this solid medium should be employed in a selective enrichment broth. Baird and van Doorne[10] described liquid Baird-Parker medium (LBP or BP broth) which is used in the resuscitation of stressed *Staph. aureus* cells, making it possible to detect their presence in low numbers using the MPN method. Results of an investigation can be obtained a day earlier compared with the standard MPN procedure. In its characteristics the medium is similar to Giolitti-Cantoni broth and it too is incubated anaerobically under a layer of oil. Blackening of the medium is used as a presumptive indicator of the presence of *Staph. aureus* and selective enrichment in LBP followed by plating on Baird-Parker agar has been found to be highly productive.

Giolitti-Cantoni broth, Baird-Parker broth and selective tryptone soya broth are described on the following pages. There are also brief descriptions of mannitol salt and salt meat broths although their direct use for enrichment of staphylococci in foods cannot be recommended.

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Table 2 – Selective and Presumptive Identification Systems used in Enrichment Media for *Staphylococcus aureus.*

Medium	Selective agents	Identification system
Giolitti-Cantoni broth	Potassium tellurite Lithium chloride Glycine Anaerobic incubation	Blackening of potassium tellurite
Liquid Baird-Parker broth	Potassium tellurite Lithium chloride Glycine Anaerobic incubation	Blackening of potassium tellurite
Salt-tryptone soya broth	Sodium chloride	–
Salt-tryptone soya broth containing sodium pyruvate	Sodium chloride	–
Mannitol-salt broth*	Sodium chloride	Mannitol fermentation
Mannitol-salt Sorbic acid broth	Sodium chloride Sorbic acid incubation at 45°C	Mannitol fermentation

*This medium does not contain a resuscitating agent and the method for its use does not incorporate a resuscitation stage.

Giolitti-Cantoni Broth

Giolitti-Cantoni Broth

Code: CM523

An anaerobic enrichment broth for *Staphylococcus aureus.*

Formula

	grams/litre
Tryptone	10.0
'Lab-Lemco' powder	5.0
Yeast extract	5.0
Lithium chloride	5.0
Mannitol	20.0
Sodium chloride	5.0
Glycine	1.2
Sodium pyruvate	3.0

pH 6.9 ± 0.2

Directions

Suspend 54.2 grams in 1 litre of distilled water and heat gently to dissolve. Dispense 19 ml amounts into 200 x 200 mm test tubes and sterilise by autoclaving at 121°C for 15 minutes. Cool rapidly then aseptically add to each tube 0.3 ml of a sterile solution of potassium tellurite 3.5% SR30.

The medium requires the addition of a 3.5% solution of potassium tellurite when there is a direct addition of 1 gram of the sample to 19 mls of broth. This level of potassium tellurite is necessary to suppress the high numbers of contaminating organisms that can be expected.

The use of diluted solution of potassium tellurite is applicable when a 1 in 10 dilution of the food sample is made.[1] In such cases the SR30 should first be diluted 1 in 10 with sterile distilled water.

The addition of 0.1% Tween 80 can be recommended in order to improve recovery of heat injured *Staph. aureus* cells e.g. from milk powder. 1 gram of Tween 80 should be added to 1 litre of CM523 prior to autoclaving.[2]

Description

Oxoid Giolitti-Cantoni Broth, a tellurite-mannitol-glycine enrichment broth, based on the formulation of Giolitti and Cantoni[3] is used for the selection and enrichment of *Staphylococcus aureus* from foodstuffs. Mannitol and sodium pyruvate are growth stimulants for staphylococci and aid detection of the organism when present in small numbers.[4]

The growth of Gram-negative lactose-fermenting bacilli is inhibited by lithium chloride and Gram-positive bacilli are inhibited by potassium tellurite in combination with glycine.

The creation of anaerobic conditions by overlaying with 2 cm of sterile paraffin wax inhibits the growth of micrococci.

Giolitti-Cantoni Broth is recommended for the detection of *Staphylococcus aureus* in dried baby milk and other weaning foods where the organism should be absent from 1 gram of test material.[6]

The medium is suitable for the examination of meat and meat products.[7] For this purpose the concentration of the potassium tellurite must be reduced to 0.35% and it is recommended that the weight of the test sample should be reduced to 0.1–0.01 gram.

Technique

The medium should be inoculated as soon as it has been cooled after autoclaving. If there is a delay in putting the medium to use it must be freed from dissolved air by immersion in free flowing steam for 20 minutes.

Inoculate 1 gram of sample material and 1 ml aliquots of a series of suitable decimal dilutions into tubes containing 19 ml of Giolitti-Contoni Broth. Two tubes are used for the sample material and for each of the dilutions. This increases the likelihood of detecting *Staphylococcus aureus* when it is present in very small numbers.

The medium is overlaid with 2 cm of molten sterile paraffin wax (melting temperature 42–44°C) and incubated for 48 hours at 35°C, examining daily. The result is considered negative for *Staphylococcus aureus* if no blackening of the medium is observed. If blackening does occur at the bottom of the tubes or there is general blackening of the medium, the broth is streaked onto a staphylococcal isolation medium, such as Baird-Parker Medium[8] CM275 and incubated at 35°C for 24–48 hours. The result is considered positive if black colonies with a narrow white margin, surrounded by a zone of clearing, are seen.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at 2–8°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923

Negative control:
Staphylococcus epidermidis ATCC® 12228

Some strains of *Staph. epidermidis* may grow and produce blackening depending on the inoculum level. Growth, if it does occur, is generally much less vigorous than that of *Staph. aureus.*

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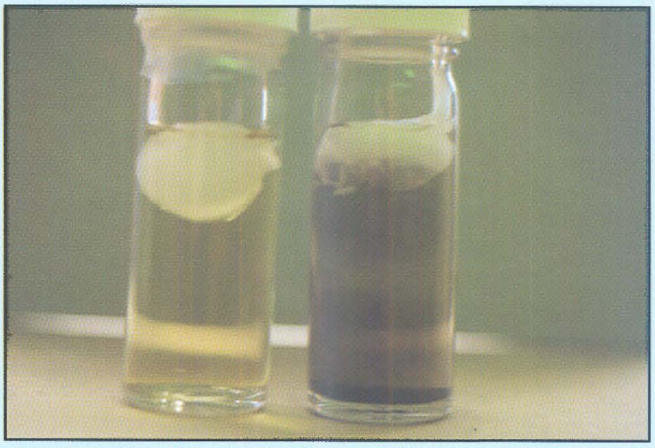

Appearance of *Staph. aureus* culture in Giolitti-Cantoni broth (right). Uninoculated medium on the left. The surface of the medium is sealed with paraffin wax to create anaerobic growth conditions.

Baird-Parker Liquid Medium (LBP Broth)

Baird-Parker Liquid Medium (LBP Broth)

Formula

	grams/ litre	*Suggested Oxoid Product*
Peptone	8	Peptone Bacteriological L37
Tryptone	2	Tryptone L42
Meat extract (Lab-Lemco)	5	Lab-Lemco Powder L29
Yeast extract	1	Yeast Extract Powder L21
Sodium pyruvate	10	
Glycine	12	
Lithium chloride	5	

After sterilisation, add potasium tellurite to a final concentration of 100 mg/litre. Diluted potassium tellurite 3.5% SR30 can be used. Egg yolk is omitted from this medium to improve visual detection of growth.

Description

LBP broth is a liquid modification of Baird-Parker agar and is used for selective enrichment of very small numbers of *Staphylococcus aureus*. It shares with Baird-Parker agar the ability to resuscitate sub-lethally injured cells.

LBP broth was developed to overcome the relatively poor performance of existing selective enrichment broths which contained a high concentration of sodium chloride and inhibited the growth of damaged cells present in pharmaceutical products for topical use. It has subsequently found a use in the investigation of foods for the presence of coagulase-positive staphylococci. The medium may be used to detect the presence or absence of the organisms and for estimation of the most probable number.

LBP broth and Baird-Parker agar are very similar in their inhibitory properties and growth of *Proteus* spp, *Bacillus* spp and coagulase-negative staphylococci may occur in LBP broth. Anaerobic incubation of the medium reduces the likelihood that micrococci will grow.

The growth of *Staph. aureus* causes blackening of LBP broth but as some other organisms that may also grow in the medium will do the same, the medium is not diagnostic. All cultures must be plated on an isolation medium and identification tests carried out.

Enrichment in LBP broth followed by plating on Baird-Parker agar modified by adding acriflavin, polymyxin B and sulphadimidine[2] was found to be better for enumeration of heat-stressed *Staph. aureus* in dried milk products than other combinations of media.[3]

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Tryptone Soya Broth

Tryptone Soya Broth

Code: CM129

When supplemented with sodium chloride and sodium pyruvate tryptone soya broth may be used for selective enrichment of *Staphylococcus aureus*.

Formula

	grams/litre
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5

pH 7.3 ± 0.2

Directions

Add 300 g to 1 litre of distilled water, mix well and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes.

Description

Tryptone Soya broth is a highly nutritious general-purpose medium for bacteria and fungi. The combined hydrolysates of casein and soya it contains provide a wide range of essential nutrients making the medium suitable for resuscitation of cells which have incurred sub-lethal damage.

Tryptone Soya broth is a versatile medium with many applications in pharmaceutical and clinical microbiology in addition to its use with foods. Because of its ability to initiate growth from very low numbers of organisms it is specified in the United States Pharmacopeia under its alternative name of Soybean Casein Digest Medium USP for sterility testing of pharmaceutical products.[1] In food microbiology it is used as the basis of a number of media for the selective enrichment of *Escherichia coli* O157[2,3] and an American method employs Tryptone Soya broth for repair and enrichment of *Staphylococcus aureus* in processed foods.[4] Following a period of incubation in non-selective Tryptone Soya broth to enable damaged cells to repair, sodium chloride is added for selective enrichment of the culture.

Tryptone Soya broth, with added sodium chloride and sodium pyruvate, is also specified in a selective enrichment procedure for detection of small numbers of *Staph. aureus* in raw food ingredients and unprocessed foods expected to contain a large population of competing species.[5]

These procedures for repair and enrichment and selective enrichment are summarised in the next column.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at room temperature.

Quality Control

Positive control:
Streptococcus pneumoniae ATCC® 6303
Staphylococcus aureus ATCC® 25923

Negative control:
Uninoculated medium

References

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Procedure 1 for use of Tryptone Soya Broth

Repair–selective enrichment procedure for *Staph. aureus* in foods.

This procedure is recommended for testing processed foods which are likely to contain small numbers of damaged cells.

50 ml of 1/10 dilution of prepared sample
⇩
50 ml of double-strength Tryptone Soya broth
⇩
Incubate 3 hours at 35°–37°C
⇩
Add 100 ml of single-strength Tryptone Soya broth containing 20% sodium chloride
⇩
Incubate 24 ± hours at 35°–37°C
⇩
0.1 ml of enrichment culture to duplicate plates of Baird-Parker agar. Spread to obtain isolated colonies. Incubate 46 ± 2 hours at 35°–37°C

Reference

Heidelbaugh, N.D., Rowley, D.B., Powers, E.M. *et al.* (1973) *Appl. Microbiol.* **25,** 55.

Procedure 2 for use of Tryptone Soya Broth

Selective enrichment procedure for *Staph. aureus* in foods.

This procedure is recommended for detecting low numbers of *Staph. aureus* in the presence of high numbers of accompanying organisms in raw food ingredients and unprocessed foods.

Decimal dilutions of prepared sample*

⇩ 1 ml	⇩ 1 ml	⇩ 1 ml
10 ml Tryptone Soya broth + 10% NaCl + 1% sodium pyruvate	10 ml Tryptone Soya broth + 10% NaCl + 1% sodium pyruvate	10 ml Tryptone Soya broth + 10% NaCl + 1% sodium pyruvate
⇨	⇩	⇦

Incubate 48 ± 2 hours at 35°–37°C
⇩
Subculture each growth-positive tube to Baird-Parker agar
⇩
Incubate 48 ± 2 hours at 35°–37°C

*The maximum dilution must be high enough not to yield growth.

Reference

AOAC (1987) *J. Assn. Off. Anal. Chem.* **70,** 393.

Salt Meat Broth

Salt Meat Broth

Code: CM94

An enrichment broth for halophilic organisms, especially staphylococci.

Formula

	grams/litre
Peptone	10.0
'Lab-Lemco' powder	10.0
Neutral heart muscle	30.0
Sodium chloride	100.0
pH 7.6 ± 0.2	

Directions

Add 2 tablets to 10 ml of distilled water in a 5/8 in. diameter test tube and soak for 5 minutes. Mix the contents and sterilise by autoclaving at 121°C for 15 minutes.

Description

Salt Meat broth is an enrichment medium for the isolation of staphylococci from grossly contaminated specimens such as faeces, particularly during the investigation of staphylococcal food poisoning. A salt meat medium will detect small numbers of staphylococci when mixed with large numbers of other bacteria.[1,2]

The medium cannot be recommended for direct enrichment of staphylococci in foods. Productivity is likely to be poor unless stressed organisms are first incubated in a non-selective medium, e.g. Tryptone Soya broth to allow resuscitation to occur before exposure to the high salt content.

The medium is suitable for the cultivation of some of the halophilic micrococci associated with hides and raw salt supplies.

It should be noted that staphylococci growing on this medium cannot be directly tested for coagulase production – they should first be subcultured on a medium which contains less salt. Blood Agar Base CM55 is recommended for this purpose.

Technique

After 24 to 48°C hours' incubation at 35°–37°C discrete colonies may be obtained by plating out a small portion of the liquid culture on Mannitol Salt Agar CM85 or Staphylococcus Medium No. 110 CM145.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium below 25°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923

Negative control:
Escherichia coli ATCC® 25922

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Mannitol-Salt-Sorbic Acid (MSSA) Broth

Mannitol-Salt-Sorbic Acid (MSSA) Broth

Formula

	grams/ litre	*Suggested Oxoid Product*
Tryptone	15.0	Tryptone L42
Yeast extract	5.0	Yeast Extract Powder L21
Sodium chloride	75.0	Sodium chloride Bacteriological L5
Mannitol	10.0	
Sorbic acid	1.5	
Thioglycollic acid	0.3	
Cystine	0.05	
Water	100 ml	
pH 6.7–7.0		

Distribute the medium in 15 ml amounts in 16 x 150 mm screw-capped tubes. Sterilise at 121°C for 15 minutes. Cool rapidly to 25°C and cap tightly until use.

Description

MSSA broth is an anaerobic liquid medium used in a two-step methodology for detection of *Staphylococcus aureus* in a Most Probable Number (MPN) procedure, followed by plating on Staphylococcus Medium 110 with added egg yolk. The medium was developed specifically to address the major concerns in isolating organisms from foods and for the testing of frozen seafoods in particular. Sorbic acid is used as the selective agent because of its activity against species of *Bacillus*, *Escherichia*, *Pseudomonas*, *Proteus*, *Micrococcus* and *Enterococcus*. The anaerobic conditions in the medium enhance inhibition of micrococci.

MSSA broth cultures are plated on modified Staphylococcus Medium 110 for confirmation of the presence of *Staph. aureus*. Incubation of the agar cultures at 45°C increases selectivity and intensifies the egg yolk reaction.

Reference

Raj, H. (1966) *Can. J. Microbiol.* **12,** 191–197.

Mannitol-Salt Broth

Formula

	grams/litre
Tryptone	17.0
Soya peptone	3.0
Sodium chloride	100.0
Dipotassium phosphate	2.5
Mannitol	2.5
Phenol red	0.025
pH 7.3 ± 0.2	

Description

A high salt content medium intended for selective isolation of staphylococci from foods. Mannitol and a pH indicator are present to assist detection of *Staph. aureus*.

Agar Media for *Staphylococcus aureus*

Staphylococcus aureus is an important cause of food poisoning. Although low numbers of *Staph. aureus* in foods are generally not considered a hazard, it is important that those present are detected because unchecked multiplication in favourable conditions to 10^6 per gram or more is hazardous. A large proportion of strains, when present in numbers of this order, produce significant quantities of enterotoxin within the food. Staphylococcal enterotoxins are highly heat-resistant and it is essential that tests for *Staph. aureus* are carried out on foods before processing by heat, as failure to detect viable cells does not mean that a food is safe.

Culture is still the most widely used procedure for detecting *Staph. aureus* in food samples. Many media have been developed for isolation and enumeration. Enrichment culture may not always be necessary before plating to solid media but, since most foods also contain large numbers of other microorganisms, selective culture media must be used in most circumstances.

In the clinical situation, culture on blood agar alone is often sufficient because *Staph. aureus* tends to be present in large numbers at infected sites. However, there are circumstances where use of selective media is desirable. Specimens taken from lesions may be contaminated with other organisms and *Staph. aureus* is often relatively sparse in the nasal flora of carriers. Thus special circumstances in clinical microbiology are very similar to the usual circumstances in food microbiology and sometimes the same agar culture procedures may be appropriate for both, particularly when examining food handlers for carrier status.

Non-selective bovine blood agar was preferred by Rammell and Howick[1] when examining cheese for the presence of *Staph. aureus* because the aids to identification used in existing media did not correlate well enough with the production of coagulase by strains present in the products they were testing. They found more reliable correlation between haemolytic colonies and coagulase test results but this reliability broke down if non-bovine blood was used.

Historically, a variety of selective agents have been employed and those found most effective have been included in more recent formulations. The number of formulae that have been developed is considerable, although in practice Baird-Parker agar is nowadays most frequently used.

In addition to their selective properties, it is desirable that media should also possess diagnostic properties. Examples are: the use together of tellurite for selectivity and egg yolk for identification in the medium of Alder and co-workers;[2] egg yolk for identification and sodium chloride for selectivity in the medium of Carontonis and Spink.[3] The medium formulated by Blair and co-workers[4] contains sodium chloride as the selective agent, mannitol which detects fermentation and plasma which detects coagulase production.

Selective and diagnostic agents are utilised in various combinations in different formulae. The list of selective agents which includes sodium azide, lithium chloride and glycine is not great but the combinations are many.

Detection of phosphatase and deoxyribonuclease activity have been used as identification tests. Selective agents and diagnostic markers used in a number of media are tabulated on page 15. An important feature missing from formulae that pre-dated Baird-Parker's medium[5] is the presence of components capable of resuscitating damaged cells on direct culture. The inclusion by Baird-Parker of sodium pyruvate in the medium was a considerable advance in this respect and is largely responsible for its superior performance.

Many workers have exploited the tolerance of *Staph. aureus* to concentrations of salt that inhibit the growth of other organisms. A concentration of 7.5% sodium chloride, combined with fermentation of mannitol as an identification marker is used in mannitol salt agar.[6] Later, Mossel and his co-workers combined salt with lactose fermentation.[7] Gunn[8] added lipovitellin from egg yolk to mannitol salt agar. Hydrolysis of this component by lipase produced by the majority of strains of *Staph. aureus* provided an additional diagnostic feature.

Staphylococcus Medium 110[9] combines selectivity using a high concentration of sodium chloride with mannitol fermentation, colony pigment and hydrolysis of gelatin as identification markers. Modifications to improve selectivity and diagnostic characteristics of this medium have subsequently been made by adding sodium azide[10] and egg yolk.[11] Gelatin hydrolysis has not otherwise been adopted in isolation media as an identification marker for *Staph. aureus*.

Selective salt-milk agars have been formulated. Two formulae, one British[12] and the other Russian,[13] have been used successfully. The media differ in containing extracts of meat or yeast and skimmed or whole milk. The concentration of sodium chloride in these media is 6.5%, not the usual 7.5%. Milk exerts a protective effect on damaged *Staph. aureus* cells assisting them to withstand this concentration of sodium chloride.

The ability of *Staph. aureus* to produce lecithinase and lipase has been recognised for many years and, in many formulae, detection of these enzymes by their effect of clearing the opacity of a suspension of egg yolk contained in the medium is used as a diagnostic marker.

Tests for other phenotypic characteristics of *Staph. aureus* have been incorporated in media as identification aids: coagulase production combined with mannitol fermentation,[14] coagulase production and reduction of potassium tellurite,[15] deoxyribonuclease production[16] and phosphatase production.[17] A modification to deoxyribonuclease medium, in which methyl green is incorporated in the medium makes it unnecessary to use acid to demonstrate DNAse activity making subculture or reincubation possible.[18]

The use of antibiotics in media for selection of staphylococci is somewhat less extensive than for some other organisms but Orth and Anderson,[14] used polymyxin to overcome a specific problem that sometimes led to false identification of isolates as *Staph. aureus*. Some Gram-negative organisms which may be present in association with staphylococci utilise the sodium citrate used to prevent blood coagulating in the production of plasma, consequently destroying its chelating properties. This leads to falsely-positive coagulase reactions due to the plasma clotting in the absence of citrate. Growth of Gram-negative organisms was inhibited by incorporating polymyxin B in the medium.

It was also observed that a proportion of *Staph. aureus* strains produce fibrinolysin which interferes with the coagulase reaction, resulting in false-negative results. This can be overcome by incorporating trypsin inhibitor in the medium.

Finegold and Sweeney,[19] faced with the need to detect staphylococci causing cross-infection in hospitals found that existing media, including that formulated by Moore[20] for the similar purpose of detecting "epidemic" strains of antibiotic-resistant staphylococci, were inadequate and added polymyxin B and cycloheximide to nutrient agar. Phenolphthalein phosphate was incorporated to detect phosphatase production. Slide and tube coagulase tests gave results identical to those from nutrient agar.

This medium could possibly be used in food manufacturing

environments although growth of spore-forming bacteria would not be inhibited. It might also be suitable in screening tests for detection of carriers of *Staph. aureus*.

A further development of polymyxin agar intended for the detection of nasal carriage of *Staph. aureus* was described by Davis and Davis[21] who added mannitol and bromocresol purple indicator to aid differentiation from other organisms that grew.

Polymyxin has been used by other workers. It has been added to phosphatase medium[11] to give it selectivity and is used with potassium tellurite in TPEY agar.[22]

Nalidixic acid and colistin are added to Columbia blood agar to make Columbia CN agar,[23] used in clinical microbiology but which might also be used for screening for carriers of *Staph. aureus*. The medium with sheep blood may assist in confirming the identity of a coagulase-positive isolate when possibly it may not be *Staph. aureus* (see page 29).

Cycloheximide is present in KRANEP agar[24] to inhibit moulds following its successful use in other formulae.

Neomycin is combined with sodium azide in a medium devised by Tatini, Hoover and Lachica.[25]

An early formula[26] used penicillin in a medium to detect penicillin-resistant *Staph. aureus* in hospitals. This proved to be unsuitable for use in food microbiology because at that time many of the strains found in foods were still susceptible to penicillin.[27] The medium contained, in addition to penicillin for selectivity, fibrinogen so that isolates could be identified simultaneously by the coagulase reaction. McDivitt and Jerome[28] used polymyxin in their fibrinogen medium and also added cycloheximide for increased selectivity when testing raw milk but found that the medium was inferior to the tube test for identifying coagulase-positive isolates.

Antibiotics, sulphonamides and acriflavin have been used in modifications to Baird-Parker agar to improve its selectivity.[29,30] Sulphonamides are chosen to inhibit *Proteus* spp. because high salt content and high pH, which are also effective, solubilise egg yolk, preventing the egg yolk reaction from being observed.

Skorkovský[31] chose potassium thiocyanate to fulfil the need for a more effective selective agent for his medium.

Potassium thiocyanate was later also used by Sinell and Baumgart[23] as a constituent of a cocktail of selective agents incorporated in KRANEP agar in which presumptive identification is carried out by noting mannitol fermentation, the egg yolk reaction and colony pigmentation. KRANEP agar also contains lithium chloride and sodium azide which had been used by earlier workers.

Vogel and Johnson[32] utilised mannitol fermentation and coagulase production in their medium because they are described in Bergey's Manual as key characteristics of *Staph. aureus*. Experience with both coagulase-positive and coagulase-negative strains had convinced these collaborators that colony pigment was an unreliable characteristic for identifying *Staph. aureus* and although used as an identifying characteristic by other workers should be ignored. Additionally, mannitol fermentation itself did not always accompany coagulase production. Some mannitol-fermenting strains failed to give a positive coagulase test on mannitol-salt agar which at that time was extensively used. Vogel and Johnson consequently directed their work to development of a medium that would reliably and consistently grow strains that demonstrate both mannitol fermentation and coagulase production by effectively inhibiting strains that did not show both characteristics.

Glycine, an effective inhibitor of Gram-negative organisms, had earlier been used with potassium tellurite by Zebovitz and his co-workers.[33] Vogel and Johnson noted the advance and substituted potassium tellurite and glycine for sodium chloride in a mannitol medium. Blackening of colonies due to the reduction of tellurite is useful as an additional identification marker. Glycine is also advantageous as it has a secondary function of being a growth stimulant because it is an essential component of the cell wall of staphylococci.

Vogel and Johnson agar was applied successfully in predominantly American methodology but following the introduction of Baird-Parker agar,[34] it became apparent that productivity was not as great as that of the new medium. Andrews and Martin[35] modified Vogel and Johnson agar to make it as capable of resuscitating damaged cells as Baird-Parker agar. Egg yolk in Baird-Parker agar was shown experimentally by them to contribute to resuscitation in addition to its function in presumptive identification. Phosphatidyl choline (lecithin) is a major constituent of egg yolk and was included in the medium when shown to be the constituent in egg yolk important in cell resuscitation. Its presence in Vogel and Johnson agar, with meat extract for additional nutrition and catalase spread on the plate surface to inactivate toxic oxygen species, enables a significant increase in colony counts to be achieved. Deoxyribonucleic acid was also added to enable deoxyribonuclease production to be used as an additional identification test. This modification of Vogel and Johnson agar known as PCVJ agar is as productive as Baird-Parker agar but has not been widely adopted.

Many other media have been devised for detection of *Staph. aureus* in both clinical specimens and food samples. Some of the advances made in media intended for clinical purposes may be useful when examining foods and checking the hygiene of food manufacturing areas. Innes,[25] for his tellurite-egg medium, modified Ludlam's medium[36] to make it more suitable for use with foods by reducing the concentration of potassium tellurite. The modified Ludlam's medium showed greater productivity for staphylococci but there was an increase in contamination by *Bacillus* spp.

Some strains of coagulase-positive staphylococci did not always show their typical appearance on Ludlam's medium. However, it was thought to be the best then available and, despite the limitations already noted, was used by Innes as the basis of a new selective diagnostic medium. Egg yolk was incorporated because of the close correlation noted by Gillespie[37] between egg yolk and coagulase results. The opacity of egg yolk was improved by reducing the concentration of sodium chloride. The lithium chloride content of Ludlam's medium was not altered but the amount of potassium tellurite was decreased to improve productivity. The increased growth of contaminating organisms on this less selective medium was counteracted by reducing the Lab-Lemco meat extract content to reduce the nutritional quality.

There are some departures from the usual selective agents and diagnostic markers incorporated in media for *Staph. aureus*. The selective activity of triphenyltetrazolium was used by Kennedy and Barbara.[38] Haemolytic zones surrounding colonies on the selective medium of Sevel and Plommet[39] are indicative that the organism is *Staph. aureus*.

Menolasino *et al.*[40] used eosin-methylene blue agar for the identification of coagulase-positive staphylococci which grew as characteristic colourless pinpoint colonies. The medium cannot be recommended for this application because the growth of many strains is completely inhibited.

Baird-Parker originally designated his medium ETGP agar

from the initial letters of the diagnostic, selective and growth stimulating constituents it contains, these being Egg yolk, Tellurite, Glycine and Pyruvate. The medium is now commonly known as Baird-Parker or B-P agar.

In formulating the medium, Baird-Parker combined the selective agents potassium tellurite and glycine used by Zebovitz with demonstration of clearance of egg yolk around colonies of *Staph. aureus* employed by earlier workers, including Hopton[41] for his selective egg-yolk-azide medium. Zebovitz had noted the successful use by Ludlam of potassium tellurite in combination with chloride but discarded the lithium chloride and substituted glycine in the improved medium. Ludlam had poised his medium at pH 9.6 but the feature was abandoned when experience showed that it was difficult to achieve this pH value consistently. Hopton combined the selective property of sodium azide with the observation by Gillespie and Adler of the production of opacity by coagulase-positive staphylococci in a medium containing egg yolk. Baird-Parker's use of the revitalising and growth stimulatory properties of sodium pyruvate appears not to have been reported before in a medium for selective isolation of *Staph. aureus*. Catalase may be spread over the surface of the medium to supplement the effects of pyruvate.[42]

Developments in the progression towards Baird-Parker agar are summarised in Table 4.

Baird-Parker agar very successfully assists recovery of stressed *Staph. aureus* cells. It is usual for samples to be cultured directly on it without the need for prior incubation on a non-selective medium, although initial incubation on non-selective base medium was later found by other workers to improve performance.[43]

Baird-Parker agar is the plating medium frequently specified in standard methods but has been criticised for inadequate selectivity. Both Gram-positive and Gram-negative flora may grow on it, including enterococci, *Bacillus* spp., micrococci and *Proteus* spp. Some modifications made to the medium to improve selectivity are shown in Table 5.

Although Baird-Parker agar is very versatile, other media may be more effective in particular circumstances.

Stiles[44] found when evaluating media for recovery of *Staph. aureus* from cheese that, overall, mannitol-salt agar was the most reliable. Staphylococcus Medium 110 also performed well. Salt-egg yolk agar[3] and Colbeck egg yolk medium[45] were also preferable to Baird-Parker agar in this application. These findings further confirm the necessity to match culture media to the food under investigation.

The egg yolk reaction is not always reliable in Baird-Parker agar and, in any case, it only gives a presumptive identification.

All colonies suspected of being *Staph. aureus* must be tested for coagulase. Many years earlier Penfold,[46] working in the Emergency Public Health Service, had shown the efficacy of direct presumptive identification of coagulase-positive *Staphylococcus* colonies on an agar medium containing outdated human blood bank plasma but lacking other nutrients. Selective agents were not added. Several years later, Duthie and Lornez[47] and Klemperer and Haughton[25] confirmed the value of using fibrinogen as a more reliable indicator than outdated blood bank plasma.

Deneke and Blobel[48] used fibrinogen by applying it to the agar surface. Coagulase-positive colonies have a distinctive "halo" appearance when viewed under indirect illumination. The technique was found to be highly effective when used with Zebovitz[30] tellurite-glycine medium. Direct testing for coagulase in Baird-Parker agar was introduced by Stadhouders and co-workers[49] who substituted pig plasma for egg yolk. In a development fibrinogen was incorporated to improve the clarity of the coagulase reaction.[50] Beckers[51] further improved the medium by substituting rabbit plasma for pig plasma. Trypsin inhibitor is included in this rabbit plasma/fibrinogen medium and it has been necessary to reduce the content of potassium tellurite because of excessive inhibitory activity in the absence of egg yolk.[52] The correct medium constitution is achieved in commercial medium by substituting Rabbit Plasma-Fibrinogen (RPF) supplement for Egg yolk-Tellurite Emulsion.

New formulae continue to be reported. Mintzer-Morgenstern and Katzenelson[53] considered the complex formula, short storage life, poor inhibition of *Proteus* and failure of some strains to show the typical egg yolk reaction to be serious disadvantages of Baird-Parker agar. To overcome consequent difficulties the authors devised a selective medium for isolation and identification of coagulase-positive staphylococci in a single step. The medium, named Staph. 4-S medium, contains potassium tellurite, a high salt content and egg yolk. Incubation at 42°C ensures that *Proteus* is inhibited. The greater reliability of the egg yolk reaction in this medium is attributed to more effective inhibition of organisms that can interfere with it.

TPEY medium described by Crisley[21] employs the egg yolk reaction and combines potassium tellurite and polymyxin B as selective agents.

EYAA medium which contains egg yolk and sodium azide has been described by Scandinavian workers.[54] Rammell and Howick[1] preferred to return to basics with a medium that does not contain selective agents and relies on the characteristic haemolytic appearance for visual selection of *Staph. aureus*.

A medium containing salt, mannitol and plasma described by Blair and co-workers[4] is a variant of early mannitol-salt formulae.

Ruffo[55] combined plasma with bovine albumin and fibrinogen in another high-salt content medium. Lactose was included for easy differentiation of *Staph. aureus* from coagulase-negative staphylococci and micrococci in milk samples collected from mastitic cattle.

Schleifer and Kramer[56] considered potassium thiocyanate to have significant advantages as a selective agent and incorporated it in their medium formulated in 1980.

It seems likely that media will continue to be formulated, for no one medium is entirely satisfactory in all circumstances.

Some of the media here briefly reviewed are described in greater detail on the following pages.

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Table 3 – Selective Agents and Presumptive Diagnostic features employed in some Agar Media for *Staphylococcus aureus*.

Medium	Selective Agents	Diagnostic Features	Reference
Mannitol salt	Sodium chloride	Mannitol fermentation	1
Lipovitellin-salt-mannitol agar (LSM)	Sodium chloride	Mannitol fermentation Egg yolk reaction	2
Baird-Parker*	Potassium tellurite Glycine Lithium chloride	Black colonies Egg yolk reaction	3
Rabbit plasma-fibrinogen (RPF)	Potassium tellurite Glycine Lithium chloride	Coagulase reaction White or grey or black colonies	4
Vogel and Johnson	Potassium tellurite Glycine	Black colonies Mannitol fermentation	5
Improved Vogel and Johnson (PCVJ)	Potassium tellurite Glycine Lithium chloride	Black colonies Mannitol fermentation DNAse production	6
Staph 110	Sodium chloride Sodium azide[a]	Mannitol fermentation Gelatin liquefaction Pigment production Egg yolk reaction[b]	7
Staph 4S	Sodium chloride Potassium tellurite Incubation at 42°C	Egg yolk reaction Grey/dark-grey colonies	8
KRANEP	Potassium thiocyanate Lithium chloride Sodium azide Cycloheximide	Mannitol fermentation Egg yolk reaction Pigment production	9
Egg yolk-azide	Sodium azide	Egg yolk reaction	10
Columbia CNA (Staph/Strep selective medium)	Nalidixic acid Colistin sulphate	Pigment Haemolysis	11
Polymyxin-coagulase-Mannitol	Polymyxin B	Coagulase reaction Mannitol fermentation	12
DNAse medium		Deoxyribonuclease production	13
Phosphatase medium	Polymyxin B[c]	Phosphatase production	14
Tellurite-polymyxin-egg yolk (TPEY)	Potassium tellurite Polymyxin B	Egg yolk reaction Black colonies	15

Additions to Improve Performance

a Smuckler, S.A. and Appleman, M.D. (1964) *Appl. Microbiol.* **12,** 335–339.
b Carter, C.H. (1960) *J. Bact.* **79,** 753–756.
c Smith (personal communication) cited by Gilbert, R.J., Kendall, M. and Hobbs, B.C. (1969) in *Isolation Methods for Microbiologists.* Society for Applied Bacteriology Technical Series No. 3. Shapton, D.A. and Gould, G.W. (eds) Academic Press, London.

Additions can be made to Baird-Parker agar to improve selectivity. See page 18, Table 5 for details.

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Baird-Parker Agar

Baird-Parker Agar

Code: CM275

A selective and diagnostic medium for the isolation and enumeration of *Staphylococcus aureus* in foods.

Formula

	grams/litre
Tryptone	10.0
'Lab-Lemco' powder	5.0
Yeast extract	1.0
Sodium pyruvate	10.0
Glycine	12.0
Lithium chloride	5.0
Agar	20.0
pH 6.8 ± 0.2	

Directions

Suspend 63 g in 1 litre of distilled water and boil to dissolve the medium completely. Dispense into tubes or flasks and sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add 50 ml of Egg-Yolk-Tellurite Emulsion SR54. Mix well before pouring.

Prepared plates may stored at 4°C.

Description (with E-Y-T Emulsion SR54)

Baird-Parker[1] developed this medium from the tellurite-glycine formulation of Zebovitz *et al.*[2] and improved its reliability for isolating *Staph. aureus* from foods.

Baird-Parker[3] added sodium pyruvate to protect damaged cells and aid their recovery together with egg yolk emulsion as a diagnostic agent. It is now widely recommended by national and international bodies for the isolation of *Staph. aureus.*[4]

The selective agents glycine, lithium and tellurite have been carefully balanced to suppress the growth of most bacteria present in foods, without inhibiting *Staph. aureus.*

Egg yolk emulsion makes the medium yellow and opaque. *Staph. aureus* reduces tellurite to form grey-black shiny colonies and then produces clear zones around the colonies by proteolytic action. This clear zone with typical grey-black colony is diagnostic for *Staph. aureus.* On further incubation, most strains of *Staph. aureus* form opaque haloes around the colonies and this is probably the action of a lipase. Not all strains of *Staph. aureus* produce both reactions. Some strains of *Staph. saprophyticus* produce both clear zones and opaque haloes but experienced workers can distinguish these from *Staph. aureus* by the longer incubation time required.[5]

Colonies typical of *Staph. aureus* but without an egg yolk reaction should also be tested for coagulase production.[6]

Egg yolk reaction-negative strains of *Staph. aureus* may occur in some foods, especially cheese.

Smith and Baird-Parker[7] found that the addition of 50 µg of sulphamethazine per ml of medium suppressed the growth and swarming of *Proteus* species. Small numbers of *Staphylococcus aureus* could then be recovered from specimens containing mixed *Proteus* strains. Other modifications to Baird-Parker agar to improve its selectivity are shown in Table 5.

Baird-Parker and Davenport[8] showed that the recovery of damaged staphylococci was greater on Baird-Parker medium than on other recovery media tested.

Broeke[9] and de Waart *et al.*[10] found Baird-Parker medium valuable in ecological studies on foods incriminated in staphyloenterotoxicosis. 97.5% of the 522 strains of *Staph. aureus* tested, isolated from humans and food, developed characteristically and quantitatively on Baird-Parker medium.

Colony Characteristics of Typical Organisms on Baird-Parker-Egg Yolk-Tellurite Medium

ORGANISM
Staph. aureus

GROWTH
Good

COLONY
Grey-black shiny convex 1–1.5 mm diameter (18 hours) up to 3 mm (48 hours) narrow white entire margin surrounded by zone of clearing 2–5 mm

ORGANISM
Staph. hyicus

GROWTH
Variable

COLONY
Greyish-blue, becoming brown or black after 48 hours

ORGANISM
Staph. intermedius

GROWTH
Variable

COLONY
Off-white to grey-blue, becoming brown or black after 48 hours

ORGANISM
Staph. epidermidis

GROWTH
Variable

COLONY
Not shiny black and seldom produces clearing

ORGANISM
Staph. saprophyticus

GROWTH
Variable

COLONY
Irregular and may produce clearing. Wide opaque zones may be produced in 24 hours

ORGANISM
Micrococcus species

GROWTH
Variable

COLONY
Very small in shades of brown and black. No clearing

ORGANISM
Bacillus species

GROWTH
Variable

COLONY
Dark brown matt with occasional clearing after 48 hours

ORGANISM
Escherichia coli.

GROWTH
Variable

COLONY
Large brown-black

ORGANISM
Proteus species

GROWTH
Variable

COLONY
Brown-black with no clearing

ORGANISM
Yeasts

GROWTH
Variable

COLONY
White, no clearing

Technique

1 Dry the surface of agar plates for a minimal period of time prior to use.

2 With a glass spatula, spread 0.1 ml aliquots of food dilutions made up in buffered peptone water on the agar surface until dry. Up to 0.5 ml may be used on larger dishes (24 cm).

3 Incubate the inverted dishes at 35°C. Examine after 24 hours and look for typical colonies.

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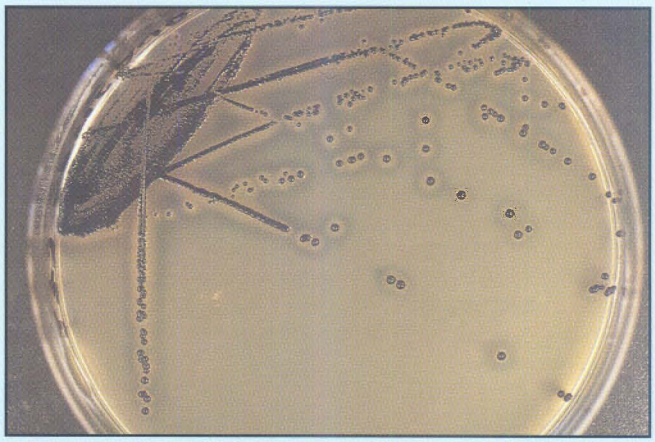

Typical appearance of *Staph. aureus* colonies on Baird-Parker agar. Note the clear zones in the egg yolk surrounding the colonies and the opaque zones of lipase activity immediately adjacent to the colonies. Not all strains show both characteristics.

Table 4 – The Progression towards Baird-Parker Agar.

Use of:			
Potassium tellurite	**Glycine**	**Egg yolk**	**Sodium pyruvate**
Ludlam (1949)			
		Gillespie (1952)	
Zebovitz (1955)	Zebovitz (1955)		
		Colbeck (1956)	
Vogel and Johnson (1960)	Vogel and Johnson (1960)		
Innes (1960)		Innes (1960) Carter (1960) Hopton (1961)	
Baird-Parker (1962)	Baird-Parker (1962)	Baird-Parker (1962)	Baird-Parker (1962)

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Hopton, J. (1961) *J. Appl. Bact.* **24,** 121.
Innes, A.G. (1960) *J. Appl. Bact.* **26,** 152–158.
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Table 5 – Modifications that have been made to Baird-Parker Agar to Improve Selectivity and Presumptive Identification.

Additional selective agent	**Purpose**	**Reference**
Sulphamethazine	Inhibition of *Proteus* spp.	1
Acriflavin	Inhibition of coagulase-negative staphylococci (CNS) and enterococci	2
Polymyxin B Sulphonamide }	Act synergistically to inhibit CNS	3
Agar overlay	Impedes growth of micrococci	4
Incubation at 42°C		
	Additional or alternative diagnostic features	
	Phosphatase production coagulase reaction	5 6

References

1 Smith, B.A. and Baird-Parker, A.C. (1964) *J. Appl. Bact.* **27,** 78–82.
2 Devriese, L.A. (1981) *J. Appl. Bact.* **50,** 351–357.
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Baird-Parker RPF Agar (rabbit plasma-fibrinogen agar)

An alternative to Baird-Parker Agar for presumptive identification of *Staph. aureus*

Basal Agar

Baird-Parker Agar base CM275.

RPF Supplement SR122

This supplement replaces egg-yolk in Baird-Parker agar for use when investigating food samples for the presence of strains of *Staph. aureus* that may show little or no egg yolk reaction. Potassium tellurite is contained in the supplement.

Formula

(per vial sufficient for 100 ml medium)

Bovine fibrinogen	0.375 g
Rabbit plasma	2.5 ml
Trypsin inhibitor	2.5 mg
Potassium tellurite	1.5 mg

Directions

To one vial aseptically add 10 ml of sterile distilled water. Turn the vial end-over-end to dissolve, taking care to avoid frothing. Dissolution is not obtained immediately and one to two hours may be required for the supplement to dissolve completely.

Aseptically add the vial contents to 90 ml of sterile Baird-Parker Agar base (Oxoid CM275) cooled to 48°C. Mix well and use immediately.

Description

A recognised disadvantage of Baird-Parker medium with egg yolk is that a coagulase test must be done to confirm that an isolate is *Staph. aureus*. Also there are some strains of *Staph. aureus* which give negative egg yolk reactions and therefore all suspicious colonies should be tested.[1,2]

Attempts were made to substitute pig plasma for egg yolk in Baird-Parker medium to obtain an *in situ* coagulase test.[3] Hauschild[4] improved the reliability of the coagulase reaction by adding bovine fibrinogen and trypsin inhibitor to the pig plasma. Beckers *et al.*[5] showed that rabbit plasma used in place of pig plasma overcame the problems of false-positive and false-negative reactions in the medium. A rabbit plasma-fibrinogen-trypsin inhibitor-tellurite supplement for Baird-Parker medium was created by Beckers *et al.*[5] which gave reliable coagulase reactions for *Staph. aureus*.

Sawhney[6] observed variation in yields of *Staph. aureus* cultures when comparing RPF supplement with egg yolk in Baird-Parker Agar base. He showed that it was necessary to reduce the tellurite level in the RPF supplement because it lacked the protective factor(s) present in egg yolk.

Oxoid RPF Supplement SR122 contains the recommended reduced level of tellurite.

The addition of RPF Supplement to Baird-Parker medium gives a translucent agar plate in which the opaque zones of the coagulase reaction can clearly be seen.

THIS IS THE DIAGNOSTIC REACTION.
DO NOT ADD EGG YOLK-TELLURITE EMULSION.

Colony Characteristics on Baird-Parker RPF Medium

It is important to note that the reduction in tellurite content means that black colonies may not be formed. *Staph. aureus* colonies may be white, grey or black, surrounded by an opaque halo of fibrin precipitation, i.e. the coagulase reaction.

Staph. epidermidis will not show the coagulase reaction at 24 hours incubation but may produce zones after 40 hours.

Most *Staph. aureus* cultures will be detected at 24 hours incubation.

Technique

Surface Inoculation Method

1 Prepare the RPF Agar plates as directed.

2 Process the food sample in a stomacher or Waring blender using the recommended sample size and diluent.

3 Make a series of decimal dilutions of the processed sample.

4 Separate plates are inoculated with 0.1 ml of the prepared samples and the decimal dilutions of them.

5 Incubate at 35°C and examine after 24 and 48 hours incubation.

6 Count all the colonies that have an opaque halo of precipitation around them. Do not limit the count to black colonies.

7 Report as the number of coagulase-positive staphylococci present per gram of food.

Pour-Plate Method

1 Prepare the RPF Agar as directed and hold at 48°C.

2 Process the food sample in a stomacher or Waring blender using the recommended sample size and diluent.

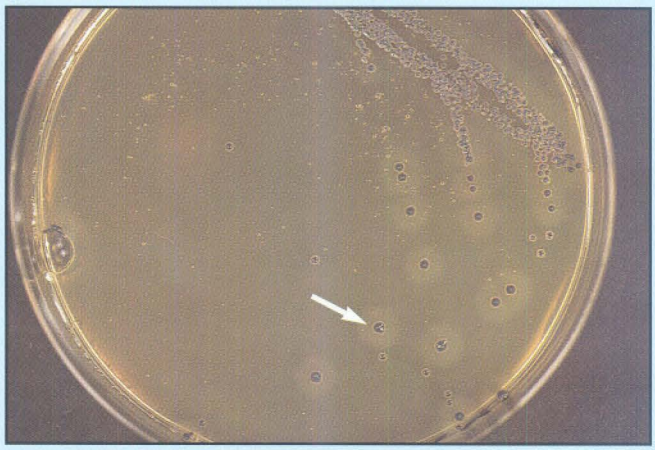
3 Add 1 ml of the prepared sample (initial suspension and subsequent decimal dilutions) into each sterile petri dish.

4 Aseptically add 20 ml of sterile RPF Agar and prepare pour-plates.

5 Incubate at 35°C and examine after 24 to 48 hours.

6 Count all the colonies that have an opaque halo of precipitation around them.

7 Report as the number of coagulase-positive staphylococci per gram of food.



Typical appearance of *Staph. aureus* colonies (arrowed) on Baird-Parker RPF agar. Note the zones of turbidity surrounding colonies. This is caused by the coagulase reaction.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the freeze-dried RPF supplement at 2–8°C. Rehydrated supplement must be used immediately and not stored.

The medium is best used freshly prepared.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923

Negative control:
Bacillus subtilis ATCC® 6633
Staphylococcus epidermidis ATCC® 155

Precautions

Regard all suspicious colonies as *Staph. aureus* regardless of negative reactions in the medium and carry out further tests.

Colonies of some contaminating organisms growing in close proximity to the coagulase-positive colonies may partially digest the coagulase halo reaction.

References

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Vogel and Johnson Agar

Vogel and Johnson Agar

Code: CM641

A selective medium for the isolation of *Staphylococcus aureus* from clinical specimens and food.

Formula

	grams/litre
Tryptone	10.0
Yeast extract	5.0
Mannitol	10.0
Dipotassium phosphate	5.0
Lithium chloride	5.0
Glycine	10.0
Phenol red	0.025
Agar	16.0

pH 7.1 ± 0.2

Directions

Suspend 61 grams in 1 litre of distilled water and bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and add 5.7 ml of sterile 3.5% Potassium tellurite solution SR30 (equivalent to 20 ml of 1% Potassium tellurite).

Description

Vogel and Johnson Agar, by selecting and identifying coagulase positive and mannitol fermenting strains, permits the early detection of *Staphylococcus aureus* from heavily contaminated foods and clinical specimens. It corresponds to the specification of the United States Pharmacopoeia[1] in terms of its formula.

Vogel and Johnson[2] modified the Tellurite Glycine Agar formula of Zebovitz *et al.*[3] by doubling the mannitol concentration to 1% (w/v) and adding Phenol Red as a pH indicator. The enhanced fermentation reaction which occurs as a result of the increase in mannitol content is clearly indicated by the development of yellow zones surrounding the colonies.

Staph. aureus is able to reduce tellurite to metallic tellurium resulting in growth as black colonies.

During the first 24 hours of incubation contaminating organisms are almost completely inhibited by tellurite, lithium chloride and the high glycine concentration. Virtually all the organisms that grow in this time are coagulase-positive.

Organisms that grow as black colonies surrounded by a yellow zone after incubation at 35–37°C for 24 hours may be presumed to be *Staph. aureus*.

Prolonged incubation may result in the growth of black coagulase-negative colonies and if these organisms also ferment mannitol they may be falsely identified from their appearance as *Staph. aureus*. In these circumstances further tests of identity should be carried out before concluding that the organism is *Staph. aureus*.

Techniques

Food samples

1 Dry the surface of the plates.

2 With a glass spatula spread from 0.1 to 1.0 ml of diluted food (macerated in 0.1% Peptone Water) over the surface of each well dried plate.

3 Incubate at 35–37°C and examine after 24 and 48 hours.

Clinical Specimens

1 Dry the surface of the prepared plates.

2 Inoculate directly with the specimen.

3 Incubate at 35–37°C and examine after 24 and 48 hours.

Colonial Appearance

Staphylococcus aureus appear as black, convex shiny colonies surrounded by a yellow zone.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at 2–8°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923

Negative control:
Escherichia coli ATCC® 25922

Precautions

Do not heat the medium after the addition of potassium tellurite

All presumptive *Staph. aureus* colonies should be confirmed with further tests.

References

1 United States Pharmacopoeia XXI (1985) *Microbial Limit Tests. Rockville. Md.*

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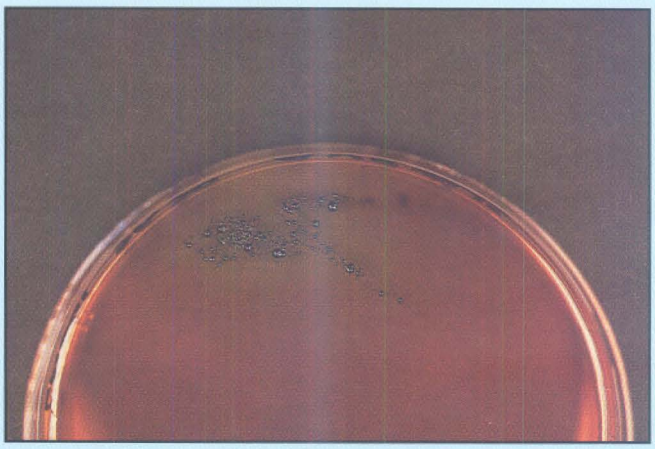


Typical appearance of *Staph. aureus* colonies on Vogel and Johnson agar. Note the yellowing of the medium indicating mannitol fermentation.

Phosphatidyl Choline Vogel and Johnson Agar (PCVJ Agar)

A modification of Vogel and Johnson agar with improved productivity.

Formula

	grams/ litre	*Suggested Oxoid Product*
Tryptone	10	Tryptone L42
Yeast extract	5	Yeast Extract Powder L21
Meat extract	5	Lab-Lemco Powder L29
Potassium phosphate	5	
Mannitol	10	
Lithium chloride	5	
Glycine	10	
Phenol red	0.025	
Phosphatidyl choline	2	
Deoxyribonucleic acid	2	
Agar	16	Agar Bacteriological L11
Potassium tellurite 1% w/v	10 ml	Potassium tellurite 3.5% SR30

pH not stated

780 units of catalase are spread over the surface of each plate before inoculation of the samples.

Description

Vogel and Johnson agar was modified by Andrews and Martin[1] to improve performance and make it comparable to Baird-Parker Agar. The unmodified medium is less productive than Baird-Parker Agar because of its inferior ability to resuscitate injured cells. Consequently the injured cells are unable to develop into colonies.

A preliminary investigation into factors that are responsible for the superiority of Baird-Parker Agar over Vogel and Johnson Agar had shown that egg yolk contributed significantly to cell resuscitation in addition to its role in presumptive identification of *Staph. aureus*. Further experimentation demonstrated that phosphatidyl choline (p.c., lecithin) which comprises approximately 25% of the content of egg yolk was responsible. Because addition of whole egg yolk was undesirable due to the opacity it gave to the medium, levels of phosphatidyl choline similar to those present in the quantity of egg yolk used in Baird-Parker Agar were added. Catalase, spread over the surface of the agar plates a short time before inoculation with the test samples is a further aid to resuscitation.

Deoxyribonucleic acid was incorporated in the PCVJ formula so that deoxyribonuclease production can be detected.

This provides a further test for presumptive identification of *Staph. aureus*. Zones of clearing that form around DNAse-positive colonies after flooding the plates with hydrochloric acid to precipitate unhydrolysed DNA are seen very easily on the clear medium.

The final improvement made to Vogel and Johnson Agar was the addition of meat extract to increase the nutritional content.

Despite the improved performance shown by the modifications to Vogel and Johnson agar Baird-Parker Agar remains the medium of choice and is widely specified in standard methods for detection of *Staph. aureus* in foods.

Reference

1 Andrews, G.P. and Martin, S.E. (1978) *J. Food Prot.* **41,** 530–532.

Mannitol Salt Agar

Code: CM85

A selective medium for the isolation of presumptive pathogenic staphylococci. Most other bacteria are inhibited, with the exception of a few halophilic species.

Formula

	grams/litre
'Lab-Lemco' powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

pH 7.5 ± 0.2

Directions

Suspend 111 g in 1 litre of distilled water and bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

Description

A selective medium prepared according to the recommendations of Chapman[1] for the isolation of presumptive pathogenic staphylococci. Most other bacteria are inhibited by the high salt concentration with the exception of some halophilic marine organisms. Presumptive coagulase-positive staphylococci produce colonies surrounded by bright yellow zones, whilst non-pathogenic staphylococci produce colonies with reddish-purple zones.

Mannitol Salt Agar is recommended for the detection and enumeration of coagulase-positive staphylococci in milk,[2] in food[3] and other specimens.[4]

Oxoid Mannitol Salt Agar has been used for the examination of meat and fish.[5,6,7,8,9]

The addition of 5% v/v Egg Yolk Emulsion SR47 to Mannitol Salt Agar enables the lipase activity of staphylococci to be detected as well as mannitol fermentation.[10] The high concentration of salt in the medium clears the egg yolk emulsion and lipase production is detected as a yellow opaque zone around colonies of staphylococci which produce this enzyme.

Technique

Heavily inoculate the Mannitol Salt Agar plate and incubate for 36 hours at 35°C or for 3 days at 32°C – the latter is recommended by the APHA.[3]

Presumptive coagulase-positive staphylococci produce colonies with bright yellow zones whilst coagulase-negative staphylococci are surrounded by a red or purple zone. Pick off suspect colonies and subculture in a medium not containing an excess of salt (e.g. Nutrient Broth No. 2 CM67) to avoid interference with coagulase or other diagnostic tests.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared plates at 2–8°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923
Staphylococcus epidermidis ATCC® 12228

Negative control:
Escherichia coli ATCC® 25922

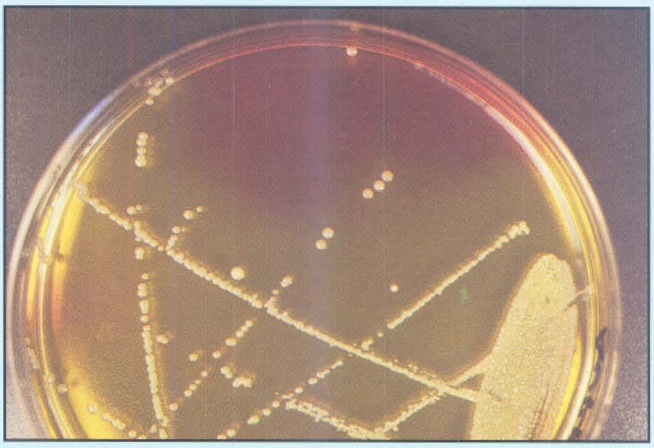
Precautions

A few strains of *Staph. aureus* may show delayed fermentation of mannitol. Negative plates should be re-incubated overnight before discarding.

Presumptive *Staph. aureus* must be confirmed with a coagulase test (Staphylase Test DR595).

References

1 Chapman, G.H. (1945) *J. Bact.* **50,** 201–203.
2 Davis, J.G. (1959) *'Milk Testing' 2nd ed.,* Dairy Industries Ltd., London.
3 American Public Health Association (1966) *'Recommended Methods for the Microbiological Examination of Foods' 2nd ed.,* APHA Inc., New York.
4 Silverton, R.E. and Anderson, M.J. (1961) *'Handbook of Medical Laboratory Formulae',* Butterworths, London.
5 Barnes, Ella M and Shrimpton, D.H. (1957) *J. Appl. Bact.* **20,** 273–285.
6 Thornley, Margaret J. (1957) *J. Appl. Bact.* **20,** 286–298.
7 Bain, Nora, Hodgkiss, W. and Shewan, J.M. (1958) *DSIR, Proc. 2nd Internat. Symp. Food Microbiol.,* 1957, HMSO, London, pp. 103–116.
8 Spencer, R. (1961) *J. Appl. Bact.* **24,** 4–11.
9 Eddy, B.P. and Ingram, M. (1962) *J. Appl. Bact.* **25,** 237–247.
10 Gunn, B.A., Dunkelberg, W.E. and Creitz, J.R. (1972) *Am. J. Clin. Path.* **57,** 236–238.



Typical appearance of *Staph. aureus* colonies on Mannitol-Salt agar.

Staphylococcus Medium No. 110

Staphylococcus Medium No. 110

Code: CM145

A selective medium for the isolation and differentiation of pathogenic staphylococci based on salt tolerance, pigmentation, mannitol fermentation and gelatin liquefaction.

Formula

	grams/litre
Yeast extract	2.5
Tryptone	10.0
Lactose	2.0
Mannitol	10.0
Sodium chloride	75.0
Dipotassium hydrogen phosphate	5.0
Gelatin	30.0
Agar	15.0

pH 7.1 ± 0.2

Directions

Suspend 150 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Disperse the precipitate by gentle agitation before pouring.

Description

Staphylococcus Medium No. 110 (Chapman[1,2]) is a selective medium for isolation and differentiation of pathogenic staphylococci on a basis of salt tolerance, pigmentation, mannitol fermentation, and gelatin liquefaction. Pathogenic staphylococci (coagulase-positive) are able to grow on the high-salt mannitol medium to form orange colonies which give positive reactions for acid production and gelatin liquefaction.

Stone[3] suggested that gelatinase activity was indicative of food poisoning strains but Chapman *et al.*[4] reported that typical food poisoning staphylococci should also produce an orange pigment, be haemolytic, be coagulase-positive, and ferment mannitol. Chapman[5] showed that incubation at 30°C produced deeper pigmentation and no interference with the Stone reaction (gelatin liquifaction) or with acid production from mannitol – both of the latter being about as intense as at 35°C.

Smuckler and Appleman[6] made Staphylococcus Medium No. 110 selective for the determination of coagulase-positive staphylococci in meat pies containing large numbers of *Bacillus* species, by the addition of sodium azide 0.75 mM (4.875 grams per litre).

Staphylococcus Medium No. 110 is formulated according to the APHA[7] and AOAC[8] specifications. Carter[9] modified the medium by adding egg yolk (5% v/v SR47) so that the characteristic egg yolk reactions of staphylococci can be seen.

Technique

Streak or smear the Staphylococcus Medium No. 110 plate with the specimen and incubate for 43 hours at 35°C or for 48 hours at 30°C. Pigmented colonies are a deep orange colour, whilst non-pigmented colonies are white.

Acid production from mannitol is best demonstrated by adding a drop of 0.04% bromothymol blue indicator to the individual colonies; yellow indicates acid production.

Gelatin hydrolysis may be demonstrated by adding a drop of a saturated aqueous solution of ammonium sulphate or, preferably, of a 20% aqueous solution of sulphosalicylic acid to an individual colony ("Stone reaction"). A positive "Stone reaction" is denoted by the presence of a clear zone around gelatinase-producing colonies after 10 minutes contact with the reagent.

The above reactions may be conveniently performed using short sleeves, 5 mm long and 10 mm diameter, cut from polythene tubing. The sleeves act as receptacles for the reagents when placed over discrete colonies, and may be stored in 70% alcohol prior to use.

Coagulase tests should not be carried out without first subculturing in Nutrient Broth No. 2 CM67 or on Blood Agar Base CM55.

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at 2–8°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923

Negative control:
Escherichia coli ATCC® 25922

References

References are given on the following page.

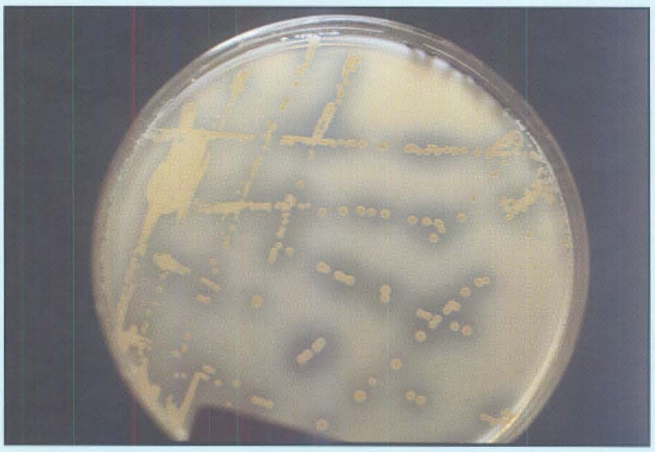
Precautions

Enterococcus faecalis may grow on this medium as tiny colonies with slight mannitol fermentation.

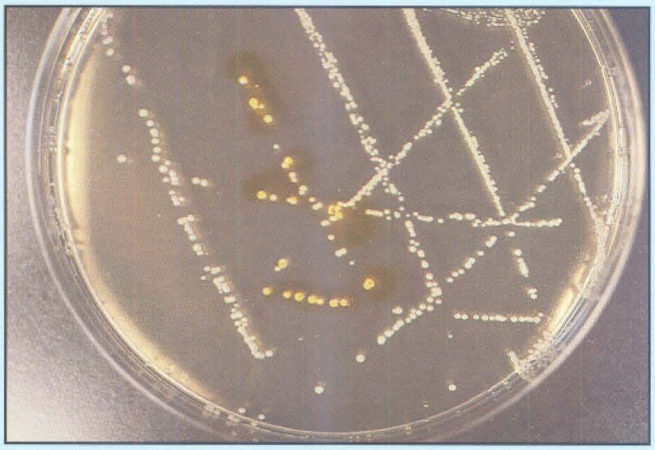
The high salt content in Staphylococcus Medium No. 110 may interfere with the coagulase reaction. Always subculture to a non-inhibitory medium before testing.

References

1 Chapman, G.H. (1946) *J. Bact.* **51,** 409–410.
2 Chapman, G.H. (1952) *J. Bact.* **63,** 147–150.
3 Stone, R.V. (1935) *Proc. Soc. Exper. Biol. & Med.* **33,** 185–187.
4 Chapman, G.H., Lieb, C.W. and Cumco, L.G. (1937) *Food Research* **2,** 349–367.
5 Chapman, G.H. (1947) *J. Bact.* **53,** 365–366.
6 Smuckler, S.A. and Appleman, M.D. (1964) *Appl. Microbiol.* **12,** 335–359.
7 American Public Health Association (1976) *Compendium of Methods for the Microbiological Examination of Foods.* APHA Inc. Washington DC.
8 Association of Official Analytical Chemists (1978) *Bacteriological Analytical Manual, 5th edn,* AOAC, Washington DC.
9 Carter, C.H. (1960) *J. Bact.* **79,** 753–756.



Typical appearance of *Staph. aureus* on Staph Medium No. 110 showing a positive gelatinase test indicated by zones of clearing around the colonies ("Stone reaction", top) and mannitol fermentation demonstrated by yellowing of bromothymol blue indicator applied to colonies (bottom).



KRANEP Agar

KRANEP Agar

A selective medium for detection and enumeration of coagulase-positive and coagulase-negative staphylococci.

Formula

	grams/ litre	*Suggested Oxoid Product*
Meat extract	1	Lab-Lemco powder L29
Yeast extract	2	Yeast extract powder L21
Peptone	5	Bacteriological peptone L37
Sodium chloride	5	Sodium chloride bacteriological L5
Potassium thiocyanate	25.5	
Sodium pyruvate	8.2	
Mannitol	5.1	
Lithium chloride	5.1	
Sodium azide	0.05	
Cycloheximide	0.041	
Agar	15.0	Bacteriological agar L11
Water	1000 ml	
pH 6.8 ± 0.2		

100 ml of egg yolk emulsion (Oxoid SR47) is added immediately before pouring.

Description

The name KRANEP given to this medium is formed from the initial letters of the principal components in the formula, i.e. Kalium-Rhodanid-Actidione-Natriumazid-Eigelb-Pyruvate.[1] KRANEP is a development of an earlier medium formulated by Skorkovský[2] which contains potassium thiocyanate and mannitol as selective and diagnostic agents. Sodium azide and cycloheximide are present to improve selectivity, sodium pyruvate as a growth stimulant and egg yolk as a second diagnostic agent.[3] The absence of potassium tellurite permits pigmented strains to be seen, providing another diagnostic feature.

The combination of selective agents inhibits a wide range of organisms including *Bacillus* spp. enterococci, *Proteus* spp., yeasts and moulds.

KRANEP agar is used for the selective enumeration of the total staphylococcal content of foods and food ingredients. The medium is particularly suitable for hygiene studies because it yields higher counts of coagulase-negative staphylococci than other selective media. KRANEP agar has been used successfully for the selective isolation of coagulase-positive staphylococci from meat products.[4]

Colony Appearances

Staphylococcus aureus

After 24 hours incubation, *Staph. aureus* forms gold, shiny, convex colonies of diameter 1.0 to 1.5 mm. After 48 hours incubation opalescent zones are present around the colonies.

Coagulase-negative staphylococci

After 24 hours, colonies appear white, shiny and convex with a diameter of 0.2 to 0.5 mm. Continued incubation will increase colony size but opalescent zones do not form.

Precautions

KRANEP agar contains potassium thiocyanate and other toxic components. It must be handled with great care using gloves, mask and eye protection. When discarding azide-containing products into drains use sufficient water to prevent accumulation of azide in the plumbing.

References

1 Sinell, H.J. and Baumgart, J. (1967) *Zent. Bl. Bakt. I. Abt. Orig.* **204,** 248–264.

2 Skorkovský, B. (1963) *Zent. Bl. Bakt. I. Abt. Orig.* **188** (4), 558–560.

3 Sinell, H.J. and Baumgart, J. (1965) *Zent. Bl. Bakt. I. Abt. Orig.* **197,** 447–461.

4 Sinell, H.J. and Kusch, D. (1969) *Arch. Hyg.* (Berlin) **153,** 56–66.

Phenolphthalein Phosphate Agar with Polymyxin (PPAP)[1]

A selective medium for the detection of phosphatase-positive staphylococci in foods.

Formula

	grams/ litre	*Suggested Oxoid Product*
Peptone	10	Peptone Bacteriological L37
Lab-Lemco meat extract	10	Lab-Lemco Powder L29
Sodium chloride	5	Sodium chloride Bacteriological L5
Agar	10	Agar Bacteriological L11

For use, add to 1 litre of base medium: 2% v/v of 0.5% w/v phenolphthalein disphosphate pentasodium salt and

Polymyxin B sulphate 125000 i.u.

Discussion

Phosphatase activity in *Staphylococcus* spp. is not limited to *Staph. aureus* although numerically it is strains of this species which are most likely to be detected. Phosphatase test culture media generally contain phenolphthalein phosphate as the substrate. Colour development in positive colonies may not always be intense and fading occurs with time, sometimes causing difficulty with interpretation. Geary and Stevens[2] substituted P-nitrophenyl phosphate for phenolphthalein phosphate to improve the test and found also that less time was needed for a result.

Baird-Parker[3] included phosphatase production in a panel of phenotypic tests in his classification scheme for staphylococci.

The principal use of phosphatase detection is in screening for presumptive identification of *Staph. aureus* using agar media.[4] Liquid media have also been applied to phosphatase detection.[5]

PPAP medium was devised for the detection of phosphatase-positive strains in foods for presumptive evidence of *Staph. aureus*. Polymyxin is included to improve selectivity. Isolates on other media may be subjected to a separate phosphatase test.

Method for Phosphatase Test[6]

Aseptically add 1 ml of 1% phenolphthalein phosphate to 100 ml of Blood Agar Base CM55. Incubate inoculated plates overnight at 35–37°C.

Expose the plate to ammonia vapour. A positive test is indicated by colonies becoming bright pink in colour.

References

1 Smith (personal communication) cited by Gilbert, R.J., Kendall, M. and Hobbs, B.C. (1969) in *Isolation Methods for Microbiologists*, Society for Applied Bacteriology Technical Series, No. 3. Shapton, D.A. and Gould, G.W. (eds) Academic Press, London.

2 Geary, C. and Stevens, M. (1989) *Med. Lab. Sci.* **46,** 291–294.

3 Baird-Parker, A.C. (1963) *J. Gen. Microbiol.* **30,** 409–427.

4 Barber, M. and Kuper, S.W.A. (1951) *J. Pathol. Bacteriol.* **73,** 65–68.

5 Pennock, C.A. and Huddy, R.B. (1967) *J. Pathol. Bacteriol.* **93,** 685–688.

6 Reference 4 above Cited in *The Oxoid Manual* 4th edition (1981) Oxoid Limited, Basingstoke UK.

Appearance of *Staph. aureus* (arrowed) on phenolphthalein phosphate agar following exposure of the medium to ammonia vapour.

Oxoid Marketing collection.

Single-Step Staphylococcus Selective Agar (Staph. 4S medium)

A simplified formula for the detection of *Staph. aureus* in foods.

Formula

	grams/ litre	*Suggested Oxoid Product*
Tryptone	4	Tryptone L42
Yeast extract	3	Yeast Extract Powder L21
Dextrose	10	Dextrose Bacteriological L71
Sodium chloride	50	Sodium Chloride Bacteriological L5
Agar	13	Agar Bacteriological L11
Water	200 ml	
50% v/v egg yolk in saline	30 ml	Egg Yolk Emulsion SR47
1% w/v potassium tellurite	3 ml	Potassium tellurite 3.5% SR30
pH not stated		

Description

Staph. 4S medium is a selective medium claimed by the originators to possess a number of distinct advantages over Baird-Parker Agar. However, it has the admitted disadvantage of severely limiting recovery of damaged *Staphylococcus* cells. During development it was seen that addition of sodium pyruvate did not improve performance but incubation of food samples in brain-heart infusion broth for 3 hours at 37°C before plating on Staph. 4S medium was found to facilitate complete recovery.

Selectivity is achieved using a combination of sodium chloride and potassium tellurite and enhanced by incubation at 42°C which inhibits the growth of *Proteus* species.

Egg yolk is included and production of a zone of opacity around colonies is used as a diagnostic feature.

Coagulase-positive staphylococci grow as small grey to dark-grey colonies surrounded by a sharply-defined dense zone of white opacity. Colonies showing this characteristic appearance are transferred to nutrient agar and a tube coagulase test done on the resultant growth.

Advantages claimed for Staph. 4S Agar are:

1 Relative simplicity of the formula.

2 Greater stability during storage.

3 Inhibition of *Proteus* species.

4 Improved diagnostic appearance of colonies with a consequent reduced necessity for checking the identity of colonies presumed to be *Staph. aureus*.

Despite the stated advantages of Staph. 4S Agar Baird-Parker Agar remains the medium of choice in standard methods.

Reference

Mintzer-Morgenstern, L. and Katzenelson, E. (1982) *J. Food Prot.* **45,** 218–222.

Tellurite-Polymyxin-Egg Yolk Agar (TPEY)

For the detection of *Staph. aureus* in baker's confectionery products.

Formula

for basal medium	grams/ litre	*Suggested Oxoid Product*
Tryptone	10.0	Tryptone L42
Yeast extract	5.0	Yeast Extract Powder L21
Sodium chloride	20.0	Sodium Chloride Bacteriological L5
Lithium chloride	2.0	
Mannitol	5.0	
Agar	18.0	Agar Bacteriological L11
Distilled water	900 ml	
Supplements		
Egg yolk emulsion 30% v/v	100 ml	Egg Yolk Emulsion SR47
Polymyxin B	40 µg/ml	
Potassium tellurite 1% w/v	10 ml	Potassium Tellurite 3.5% SR30

Directions

Autoclave the basal medium at 121°C for 15 minutes. Cool to 50–55°C. Add 100 ml of 30% v/v egg yolk emulsion, 0.4 ml of a 1% w/v sterile-filtered solution of polymyxin B and 10 ml of a sterile 1% solution of potassium tellurite.

Description

This medium was claimed by the authors to be superior to other media for the isolation of *Staphylococcus aureus* from foods but the formulae it had been compared with and the advantages claimed for TPEY agar were not stated.

In the work reported, TPEY agar had been used in an investigation into the multiplication of *Staphylococcus aureus* in synthetic cream fillings and pies.

Reference

Crisley, F.D., Angelotti, R. and Foter, M.J. (1964) *Pub. Hlth. Rep.* **79,** 369–376.

Columbia CNA Agar

BASE MEDIUM

Columbia Blood Agar Base

Code: CM331

A selective medium for staphylococci and streptococci of the type described by Ellner[1] and subsequently named Columbia CNA Agar can be made by adding Oxoid Staph/Strep supplement SR70 to Columbia Blood Agar Base CM331.

Formula

	grams/litre
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar	10.0
pH 7.3 ± 0.2	

Directions

Suspend 39 grams in 1 litre of distilled water. Boil to dissolve the medium completely. Sterilise by autoclaving at 121°C for 15 minutes.

Staph/Strep Selective Supplement

Code: SR70

For the selective isolation of *Staphylococcus aureus* and streptococci from clinical specimens or foods.

Vial content (each vial is sufficient for 500 ml of medium)

Nalidixic acid	7.5 mg
Colistin sulphate	5.0 mg

Directions

Make up and sterilise Oxoid Columbia Blood Agar Base CM331 and cool to 50–55°C. To each 500 ml of medium add 25 ml Defibrinated Horse Blood SR50 and the contents of one vial of supplement SR70, reconstituted by the addition of 5 ml of 95% ethanol. Mix gently and pour into petri dishes.

Description

Columbia CNA Agar can be prepared quickly and conveniently as and when required. Because the antibiotics contained in the supplement are freeze-dried they always show optimal activity at the time of use.

The supplemented Columbia Agar is inhibitory to *Staph. albus* and *Micrococcus* spp. as well as Gram-positive and Gram-negative rods. It suppresses growth of *Proteus*, *Klebsiella* and *Pseudomonas* species while permitting unrestricted growth of *Staph. aureus*, haemolytic streptococci and enterococci.

Columbia CNA Agar may be use for culturing specimens for *Staph. aureus* in the investigation of food handlers for carriers.

Technique

The medium is inoculated in the normal way and incubated aerobically at 35°C for 18 hours.

DO NOT INCUBATE IN CARBON DIOXIDE

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared medium at 2–8°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923
Streptococcus pyogenes ATCC® 19615

Negative control:
Escherichia coli ATCC® 25922

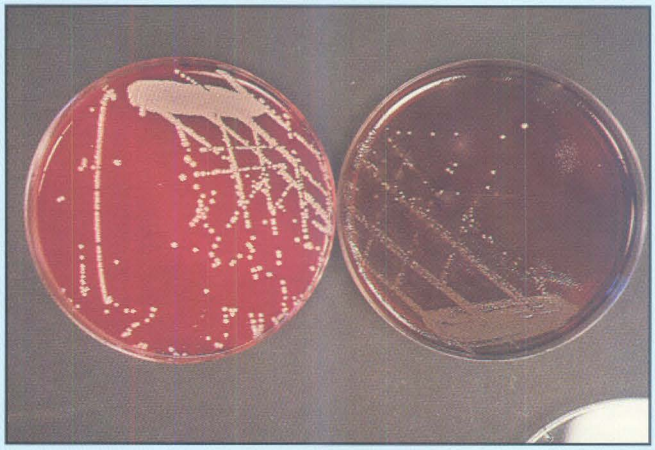
Precautions

Incubation in a CO_2-enriched atmosphere will cause inhibition of *Staphylococcus* growth.[2] If it is necessary to incubate plates in such an atmosphere Staph/Strep Supplement SR70 should not be used. All suspected *Staphylococcus* and *streptococcus* colonies should be further investigated to confirm their identity. The Staphylase Test DR595 and the Streptococcal Grouping Kit DR585 are useful for these purposes.

References

1 Ellner, P.D., Stoessel, C.J., Drakeford, E. and Vasi, F. (1966) *Tech. Bul. Reg. Med. Technol.* **36,** No. 3.

2 Morton, C.E.G. and Holt, H.A. (1989) *Med. Lab. Sci.* **46,** 72–73.



Typical appearance of *Staph. aureus* on Columbia CNA agar (left). Note inhibition of *Proteus* that is able to grow on non-selective medium (right).

Coagulase and Coagulase Testing

The detection of coagulase has emerged as the most reliable and simply performed test to distinguish pathogenic from non-pathogenic strains of staphylococci. Loeb[1] first described the formation of a coagulum or clot in a culture of *Staphylococcus aureus* growing in dilute mammalian blood plasma and, a few years later, Much[2] demonstrated the ability of *Staph. aureus* to form clumps of cells when colonies taken from solid culture medium are emulsified in plasma on glass slides. Dorang[3] drew attention to the practical significance of this behaviour of *Staph. aureus* and after these early observations Birch-Hirschfeld[4] reported advances concerning the characteristics of coagulase and its detection.

Following recognition that detection of coagulase is valuable in determining the pathogenicity of isolates, test procedures were further refined.[4,5,6] Growth as pigmented colonies and production of haemolysin, were by common consent, associated with pathogenesis but Chapman, with his co-workers, observed that coagulase-producing strains are usually pathogenic regardless of pigment and haemolysis.[7]

Results of the coagulase test indicate the presence or absence of an extracellular protein able to clot rabbit or human blood plasma prepared using the anticoagulants sodium citrate or potassium oxalate and later, EDTA (ethylenediamine tetracetic acid). This test is most simply performed in small tubes. The clumping that occurs on glass slides results from precipitation of fibrinogen around the bacterial cells.

Coagulase is not an enzyme: its action is to bind to prothrombin whereupon the complex becomes proteolytically active resulting in fibrin production.[8] Thus prothrombin is activated in the clotting mechanism without conversion to thrombin.

Cadness-Graves and colleagues[9] observed that the clumping of staphylococci in the slide test correlated well with the formation of clots in the tube test and confirmed that the demonstration of coagulase is a reliable means of identifying pathogenic strains. Berger[10] assumed that both tests were detecting the same substance and at the same time gave to it the name Clumping Factor when detected in tests performed on slides. Linsell and Gorill[11] subsequently challenged this assumption when they found that plasma that had been heated or treated with thiomersalate gave slide test-positive and tube test-negative results. Later, Duthie[12] presented evidence for the existence of two forms of coagulase in a report showing that tube and slide coagulases have different modes of action. Bound (slide) coagulase was stated to be antigenically distinct from free (tube) coagulase and is released on lysis of *Staphylococcus* cells. Duthie claimed that bound coagulase acts directly on the fibrinogen of certain animal species and free coagulase activates prothrombin to form a thrombin-like product. The scientific literature now commonly refers to two forms of coagulase: free coagulase which is detected in the tube test and bound coagulase (which may be given the alternative name Clumping Factor) which is detected in the slide test and in commercial card tests. However, there is now genetic evidence that bound coagulase of *Staph. aureus* is not the same as clumping factor and, further, that it is not responsible for adherence of cells to fibrin.[13]

The term Bound Coagulase is uneasily accepted; the opinion has been expressed that it is misleading and properly should be kept in reserve for use in the event that a cellular precursor in the production of free coagulase is ever discovered.[14]

Free coagulase is not a single substance. Eight antigenically distinct types have been identified in neutralisation tests.[15] Whatever the true relationship of bound to free coagulase, bound coagulase (clumping factor) appears not to be a secretion from *Staphylococcus* cells but is a fibrinogen receptor located on the *Staphylococcus* cell surface.

In a further development in determining the nature of coagulase, Smith and Hale[16] reported the existence of a co-factor that intensifies the action of free coagulase on fibrinogen. The speed of coagulase activity is greatly reduced if the co-factor is absent. The nature of the co-factor was unclear and the name Coagulase Reacting Factor (CRF) was give to it by Tager.[17]

Confusion still exists about the nature and relationship of the two types of coagulase but there is general agreement about how they should be detected. Free coagulase is detected by clotting of plasma in a tube test; bound coagulase (otherwise known as clumping factor) is demonstrated by the clumping of cells of staphylococci in the presence of plasma on slides or the cards used in commercially-produced tests.

The complexities of coagulase contrast with the simplicity of the methods for its detection. Coagulase production by staphylococci commences in the lag phase of the growth cycle and continues throughout the logarithmic phase.[18] In order to demonstrate clotting of plasma, cells of the test organism are mixed with diluted plasma in small test tubes and incubated at 35–37°C. Several hours are generally required for a clot to form.

Norwegian workers[19] have described a direct test for coagulase in cultures of milk samples taken from the quarters of cattle with subclinical mastitis. The method showed sensitivity of nearly 90% and specificity of 100% for recognition of *Staph. aureus* when compared with plating on blood agar. Samples are mixed with citrated rabbit plasma and incubated. Minimal facilities are required, enabling testing to be carried out on the farm.

The slide test is done by making a heavy suspension in saline from colonies growing on a solid medium and mixing this suspension with plasma on a glass slide. A positive test shows clumping of the cells which is virtually instantaneous at ambient temperatures. The slide test generally should not be performed directly from selective media, although testing from Baird-Parker Agar is satisfactory. For other selective media, colonies suspected to be *Staph. aureus* should be subcultured on non-selective agar media or may be performed satisfactorily from DNAse agar before addition of acid to demonstrate deoxyribonuclease activity. False-negative or delayed-positive results may occur if the organism suspension is too dilute. Doubtful reactions can generally be resolved if additional cells from the colony under test are rubbed into the reaction mixture.

The method for performing commercial tests is very similar to that for tests on glass slides.

A variation on the test that eliminates the need to transfer growth to a slide has been reported.[20] A drop of plasma is used to emulsify a colony growing on a solid medium. The test is no more accurate than the slide test and auto-agglutination would not be recognised. This apparent simplification of technique appears to have found few followers.

It has been recommended that a coagulase-negative control culture is included when using rapid agglutination tests because, in tests with clinical isolates, false-positive results have been associated with strains of *Staph. saprophyticus*.[21] This species is unlikely to be isolated in food microbiology methods as the media used are inhibitory.

Penfold[22] reported the direct detection of coagulase in a medium to which plasma had been added. This approach has been followed by other workers[23,24] and has subsequently been adopted in media used in food microbiology for isolation of *Staph. aureus*.

A method that enables simultaneous detection of free and bound coagulase has been described[25] that uses an albumin-plasma-fibrinogen semi-solid agar culture medium and a fibrinogen semi-solid medium as a control. The formation of diffuse colonies in the albumin-plasma-fibrinogen agar is stated to indicate that free coagulase has been produced and compact colonies in the fibrinogen agar show the presence of bound coagulase. A later modification to the medium enabled mannitol fermentation to be detected as well.[26]

Alternative methodology that dispenses with plasma to detect the staphylocoagulase of *Staph. aureus* utilises the chromogenic substrate D-phe-pro-arg-β-naphthylamide.[27] Further work is required to establish the usefulness of this new technology.

The clumping factor, although regarded as distinctly different from coagulase, is actually very similar.[28] Despite this similarity, strains exist that are free coagulase-positive and bound coagulase-negative or vice-versa. For tests that are intended to detect bound coagulase, commercial products, e.g., Oxoid Staphytect Plus that additionally detect protein A produced by *Staph. aureus* are valuable because strains that lack bound coagulase will still clump if protein A is present, enabling a correct result to be obtained. The slide test is generally suitable for routine identification purposes, particularly when test speed, simplicity and cost are important, although, when results are equivocal, greatest reliance should be placed on the tube test.

There are limitations to both tube and slide tests. In addition to the rare absence of either bound or free coagulase which can cause false identification of a strain, there are a number of factors that can lead to false-positive or false-negative results with strains that are not atypical.

The tube test may not always detect coagulase for one or more of a variety of reasons:

False-negative results may occur because plasma may fail to react due to the presence in the plasma of antibodies to coagulase.

Plasma may be deficient in fibrinogen. This possibility is particularly likely when outdated human blood bank plasma is used for the test.

Some strains test more satisfactorily if pig plasma is used instead of rabbit plasma.[29]

The strain of *Staphylococcus* under test may produce fibrinolysin which destroys fibrin, thus preventing the coagulase reaction.

Other factors which inhibit the activity of coagulase may also be present and high concentrations of sodium chloride in culture media may interfere by converting fibrinogen to a substance that is unable to clot.

False-positive results can occur when using citrated plasma contaminated with organisms that utilise citrate, e.g. *Enterococcus* spp. As a result, calcium may cease to be chelated by the citrate and again become available to take part in the clotting mechanism. EDTA can be added to citrated plasma to reinforce the anticoagulant activity[30] or alternatively potassium oxalate can be used to produce plasma. EDTA is now commonly used alone to produce plasma for coagulase testing.

False results may be given by slide tests: The suspension of organisms may not be heavy enough and/or the strain may lack clumping factor.

Infrequently, auto-agglutination of the test organism may occur. Other identification methods may need to be applied if this happens, although experience has shown that auto-agglutinable strains are likely not to be *Staph. aureus*.

The plasma used may be of poor quality or from an inappropriate animal species. Sheep and guinea pig plasma were reported by Duthie[12] not to clump staphylococci.

False identification can be avoided by performing a Gram-stain and catalase test on isolates before carrying out the coagulase test. If a slide test is unexpectedly negative, a tube test should always be done to verify the result.

Commercial products for the detection of coagulase are controlled for factors that cause false results and are highly reliable.

There is not an exclusive association between coagulase-positive staphylococci and production of enterotoxin. Some coagulase-negative species are enterotoxigenic and consequently coagulase-negative food isolates should receive greater consideration than they sometimes do. If there is any suspicion about the enterotoxigenic potential of a coagulase-negative isolate, then other tests which have shown good correlation with enterotoxin production, such as tests for protein A and thermonuclease, should be performed to ensure that the isolate is not a strain of *Staph. aureus* that has failed to produce coagulase. Commercial tests such as Oxoid Staphytect Plus which detect both bound coagulase and protein A are suitable and, for added confidence, it is often possible simply and economically, to add thermonuclease or deoxyribonuclease testing to the routine identification scheme.

Now that more precise discrimination between closely related strains is possible, it is apparent that *Staph. aureus* is not the only species that produces coagulase. *Staph. hyicus*, a species associated with pigs, poultry and, less often, bovines may be present in foods. This species produces coagulase which is detectable in the tube test but many strains lack clumping factor and consequently give negative slide-test results.

Staph. hyicus shows better clot formation if pig plasma is used and may be differentiated from *Staph. aureus* by biochemical test results although the different results for slide and tube coagulase tests are often enough to separate the species.

Staph. intermedius is coagulase-positive but as it is not generally associated with domestic animals or with humans, it is less likely to be present in foods. Growth occurs on Baird-Parker Agar but the species does not actively reduce potassium tellurite and consequently the colonies are whitish but will darken on extended incubation. White colonies are unlikely to be tested for coagulase but if *Staph. intermedius* is suspected, it can be identified by its metabolic activities.

It may on occasion be necessary to distinguish between *Staph. aureus* and *Staph. hyicus* cultures as both species are food-associated and produce coagulase. Results of key identification tests which also separate both species from *Staph. intermedius* are shown in Table 7.

Staph. schleiferi is subdivided into two subspecies: subsp. *schleiferi* which is tube coagulase-negative and clumping factor-positive and subsp. *coagulans* which shows these characteristics in reverse. However, a report[31] of production by some strains of subsp. *schleiferi* of a pseudocoagulase detectable in the tube test has complicated the identification of the two subspecies. Although clotting occurs, this would appear to be caused not by coagulase but by a separate factor which can be inactivated by protease inhibitors and anticoagulants. Similar strains could be misidentified as *Staph. aureus*, particularly as *Staph. schleiferi* is in many ways very similar. However, the *Staph. schleiferi* strains were isolated from non-enteric clinical cases and similar organisms would probably have little significance in food microbiology.

The species named above are animal-associated species that

commonly produce coagulase but some others associated with humans and animals may do so less reliably because coagulase or pseudocoagulase may be produced by a number of species, ICMSF recommend that isolates are recorded as *Staph. aureus* only if they give strong and rapid results in coagulase tests.[32]

Oxoid coagulase test kits are described on the following pages. Staphylase DR595 tests for coagulase alone; Staphytect Plus DR850 for coagulase, protein A and the capsular polysaccharide found in multi-antibiotic-resistant strains of *Staph. aureus* (MRSA).

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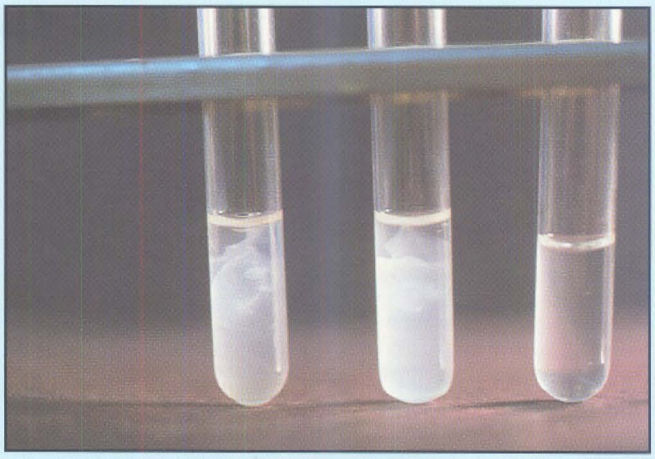
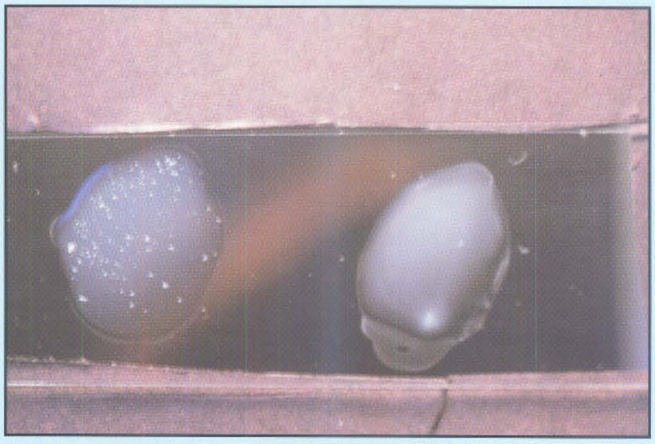


Positive tube-coagulase tests (left and centre). Negative test on the right.



Positive slide-coagulase test (left). Note the clumps of cells suspended in the plasma. A negative test showing a smooth suspension of cells in plasma is on the right.

Table 6 – Animal Associated – *Staphylococcus* species that are Positive for Coagulase, Clumping Factor and Thermonuclease Tests.

Species	Coagulase	Clumping Factor	Thermonuclease
Staph. aureus	+	+	+
Staph. intermedius	+	+*	+
Staph. hyicus	+*	–	+

*Up to 90% of strains.

Table 7 – Differentiation of Coagulase-positive Staphylococci.[1]

Test	*Staph. aureus*	*Staph. hyicus*	*Staph. intermedius*
Growth on modified[2] Baird-Parker Agar	Growth	No growth	No growth
Growth on P-agar +7 µg/ml. acriflavin[3a,3b]	**Growth**	**No growth**	**No growth**
Acid production from mannitol under anaerobic conditions	+	–	–
Acetoin test[4]	+	–	–
β-galactosidase	**–**	**–**	**+**

(Key tests are in bold type).

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Staphylase

Staphylase

Code: DR595

The generally accepted identifying characteristic of *Staphylococcus aureus* is the ability to produce coagulase. The presence of this coagulase (or "clumping factor") may be detected in a number of ways. The Oxoid Staphylase haemagglutination test detects the presence of coagulase through clumping of fibrinogen-sensitised sheep red blood cells.[1,2] The specificity of the reaction is ensured by a simultaneous test with a control reagent (unsensitised sheep red blood cells), which should not show a clumping reaction.

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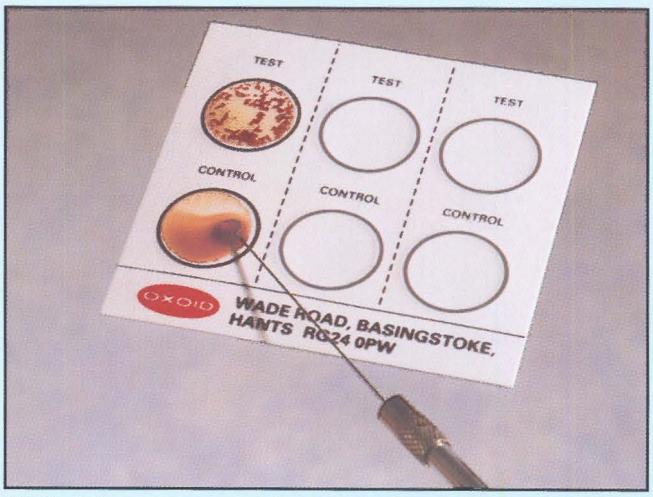


The Oxoid Staphylase test showing a positive reaction (top).

Oxoid Marketing collection.

Staphytect Plus

Staphytect Plus

Code: DR650

Staphytect Plus is a latex agglutination test for the recognition of staphylococci which possess bound coagulase (clumping factor) and protein A. It will also detect capsular polysaccharides present in multi-antibiotic-resistant strains of *Staph. aureus* (MRSA).

Staphytect Plus is available in two forms, wet reagents for the familiar latex agglutination test and the unique Dryspot™ presentation of latex reagent dried onto white disposable reaction cards. This ensures stability of the reagent until rehydrated for use with consequent greatly increased shelf life, particularly in sub-optimal storage conditions.

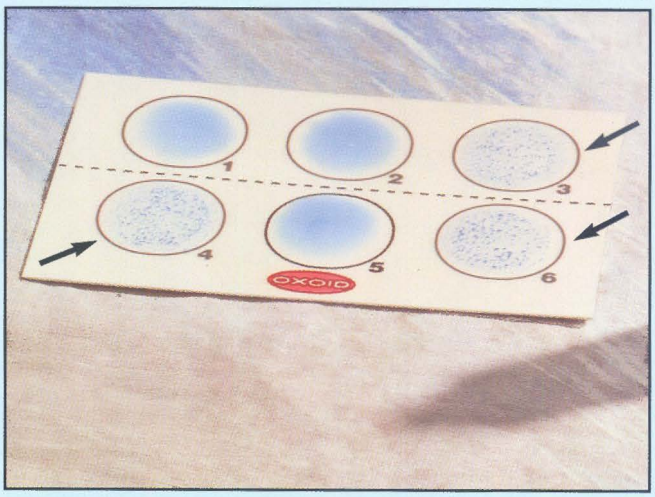
Principle of the Test

Traditionally, differentiation between coagulase-positive and coagulase-negative staphylococci has been performed with the tube coagulase test that detects extracellular staphylo-coagulase, or the slide coagulase test that detects the clumping factor (bound coagulase) present on the bacterial cell surface. Several other differentiation tests are also available including the passive haemagglutination test (Oxoid Staphylase) and the DNAse test. (See pages 35 and 39.)

It has been reported that approximately 97% of human strains of *Staphylococcus aureus* possess both bound coagulase and extracellular staphylocoagulase.

Protein A is found on the cell surface of about 95% of human strains of *Staph. aureus* and has the ability to bind the Fc portion of immunoglobulin G (IgG).

Staphytect Plus consists of blue latex particles carrying human fibrinogen, IgG and antibody to capsular polysaccharides found in MRSA strains. On mixing the latex reagent with colonies of staphylococci which have clumping factor or Protein A present on the bacterial cell surface, cross linking will occur giving visible agglutination of the latex particles. Such agglutination will occur with *Staph. aureus*. Agglutination may also occur with other species which possess clumping factor or Protein A such as *Staphylococcus hyicus* and *Staphylococcus intermedius*. If neither clumping factor nor Protein A are present in non-MRSA strains agglutination will not occur and the result will be regarded as negative. The most frequently found coagulase and Protein A-negative isolates of staphylococci are *Staphylococcus epidermidis*. The capsular polysaccharide possessed by MRSA strains may prevent agglutination when testing with kits which detect only clumping factor and protein A. Agglutination of these strains will occur when tested with Staphytect Plus.



Oxoid Staphytect-Plus showing positive (arrowed) and negative tests.

Oxoid Marketing collection.

Other Tests for Confirmation of *Staphylococcus* Species and *Staph. aureus*

Staphylococci are treated unusually in laboratory procedures in generally being classified as pathogenic or non-pathogenic on the results of a single test of species identity. This is satisfactory in clinical microbiology because *Staph. aureus* is the only coagulase-positive species associated with disease in man. It is not entirely satisfactory in food microbiology because other species of coagulase-positive staphylococci that are animal pathogens may be isolated from foods.

It is usual to assume that a positive tube and/or slide coagulase test identifies a strain as *Staph. aureus*. A high proportion of *Staph. aureus* strains produce enterotoxin and it is this pathogenic feature which is of greatest concern to food microbiologists; detection of *Staph. aureus* immediately raises questions about standards of hygiene and the safety of food. However, the situation is more complex than is commonly realised because coagulase is not exclusively produced by *Staph. aureus* and coagulase-negative strains may be enterotoxigenic. Occasionally this complicated situation may make it necessary to perform additional tests to increase confidence that an isolate has been identified correctly by the coagulase test. If necessary, strains must, as a final measure, be tested to see whether they produce enterotoxin. Interpretation of the tube coagulase test itself can be difficult. Turner and Schwartz[1] considered that any degree of clotting of the coagulase plasma on a numerical scale based on the size and firmness of the clot should be considered a positive reaction. Sperber and Tatini[2] disagreed with this interpretation following an examination of strains for species identity using tests for anaerobic fermentation of glucose, thermonuclease production and susceptibility to lysostaphin. The origin and age of the test plasma was found to influence the degree of clotting significantly and it was recommended that only tests showing formation of a complete clot that failed to be displaced when the tube was inverted should be interpreted as a positive reaction.

Although selective media in common use are generally inhibitory to micrococci, it may occasionally be necessary to ensure that an isolate is a *Staphylococcus* and not a strain of *Micrococcus* because, although the genera are superficially similar, micrococci lack the pathogenicity of staphylococci. Micrococci and staphylococci were for long considered to be members of the same genus and some confusion about the relationship of one to the other still exists. In fact the two genera share little other than a similar microscopic appearance and can most simply be distinguished by the ability of *Staphylococcus* spp., but not *Micrococcus* spp. to grow anaerobically and ferment glucose. Some species of staphylococci however, fail to ferment glucose under anaerobic conditions and, if undue emphasis is put on fermentation, they may be mis-identified as micrococci. There are other simple tests that may be done, not requiring specialist knowledge and equipment. Susceptibility to lysis by lysostaphin is one of these. Staphylococci but not micrococci are lysed by an extracellular enzyme produced by "Staphylococcus staphylolyticus" which presumably gives this organism a competitive advantage in nature. Species of staphylococci vary in their susceptibility to cell wall destruction by lysostaphin but micrococci show complete resistance.

Poutrel and Caffin[3] impregnated paper discs with lysostaphin and compared wet discs with discs that had been dried. Dried discs were preferred because of their greater stability and longer life and they identified staphylococci more accurately than the test for anaerobic fermentation of glucose. The value of the lysostaphin test had earlier been established by Schleifer and Kloos[4] in which it had been combined with a test of the ability of an isolate to produce acid aerobically from glycerol in the presence of 0.4 µg/ml of erythromycin, in a simple two-test system.

Geary and Stevens[5] were able to reduce the time needed for results of the lysostaphin test to two hours from initial isolation. They found it to be more accurate than the glycerol-erythromycin test and suggested that it is useful in both food and clinical microbiology work.

Micrococci may also be distinguished from staphylococci by culturing on a medium containing the nitrofuran furoxone.[6] Micrococci are able to grow on this medium while growth of staphylococci is prevented. Furazolidone[7] and bacitracin[8] are other antimicrobial agents that have been used to distinguish the two genera. Modified oxidase and benzidene tests that separate staphylococci and micrococci have been described by Faller and Schleifer.[9]

Micrococci and staphylococci may also be distinguished serologically.[10]

The results of tests to distinguish *Micrococcus* and *Staphylococcus* are shown in Table 8.

Occasionally it may be necessary to confirm that an isolate is not a *Streptococcus*. This is most easily done by testing for catalase produced by staphylococci but not streptococci. The testing is done by suspending a colony in 3% hydrogen peroxide on a glass slide. Immediate and vigorous bubbling is caused by staphylococcal catalase as the hydrogen peroxide is converted to water and oxygen gas.

When there is no doubt that an isolate is a species of *Staphylococcus* but doubt remains that it is *Staph. aureus* the result of a coagulase test is insufficient; further tests are necessary although a full biochemical profile may not always be required. Tests to detect nucleases are useful. *Staph. aureus* produces deoxyribonuclease (DNAse) and a heat-stable endonuclease (thermonuclease). There is close correlation between possession of these enzymes and coagulase production and particularly close correlation between thermonuclease production and the production of enterotoxin. Both DNAse and thermonuclease hydrolyse DNA. A spectrophotometric method of detection has been described[11] but microbiological methods are generally used.

Deoxyribonuclease may be detected by surface-plating on a medium containing deoxyribonucleic acid (DNA) and, after colonies have formed, flooding the agar surface with hydrochloric acid.[12] DNA is precipitated by the acid, making the medium opaque. DNAse activity is indicated by zones of clear agar around the colonies where the DNA has been hydrolysed and fails to precipitate.

Application of hydrochloric acid to DNAse medium renders the colonies non-viable and therefore sub-culture to enable further tests to be carried out is not possible after determining a positive DNAse test. This disadvantage may be overcome by incorporating in the medium metachromatic dyes which change colour as DNA hydrolyses. Smith[13] added methyl green to DNAse agar. A clear zone develops around DNAse-producing colonies without any further additions. Lachica and Deibel[14] suggested using acridine orange to make a medium on which organisms that do not produce DNAse are surrounded by dark zones of non-fluorescence when the growth is observed under fluorescent light. Schreir[15] suggested toluidine blue as an alternative metachromatic dye. The shade of blue changes as DNA is hydrolysed. Toluidine blue is most suitable for detecting DNAse in Gram-negative organisms, but, with suitable caution, its inhibitory activity towards Gram-positive organisms may be controlled so that

the dye may be used for detecting staphylococcal nucleases.

The DNAse test should not be used alone as an alternative to the coagulase test for identifying *Staph. aureus* because a high proportion of coagulase-negative strains also produce DNAse. *Staph. hyicus* too, produces DNAse.

Staph. aureus possesses a remarkably thermostable nuclease which correlates more reliably with coagulase and is a very satisfactory indicator that a strain is able to produce enterotoxin. Chesbro and Auborn[15] were the first to screen for thermonuclease activity. Lachica and co-workers[17] developed a number of methods for its detection using toluidine blue at very low concentrations in agar and broth cultures. Rayman[18] recommended detection of thermonuclease as an alternative test to perform on cultures giving doubtful coagulase reactions. Improvements have been made in methodology using toluidine blue for thermonuclease detection[19] and, despite its innate capacity to inhibit Gram-positive organisms, toluidine blue is used more commonly than methyl green for detection of staphylococcal thermonuclease.

A combined plating method and nuclease assay[20] utilises growth on Baird-Parker Agar, followed by incubation at 60°C to inactivate heat-labile nuclease before overlaying with toluidine blue-DNAse agar and noting zones of colour change surrounding colonies. This method gives extra confidence in correct identification of *Staph. aureus* if a strain does not appear typical on Baird-Parker Agar.

Lachica, Hoeprich and Franti[21] had earlier extended the usefulness of thermonuclease detection by using a microslide technique for quantitative assay in milk and broth cultures. When thermonuclease-containing preparations were placed in a well cut in the agar a pink halo was obtained as the thermonuclease diffused through the gel. The diameter of this zone was related to the time and temperature of incubation and to the concentration of the nuclease. With suitable control of the test, wide-ranging concentrations of thermonuclease could be determined in as little as 3 hours, making it possible to arrive at a rapid assessment of whether enterotoxin might also be present.

Staph. aureus is not the only coagulase-positive species; most strains of the pig pathogen *Staph. hyicus* will give a positive tube test. Test performance with this species is improved if pig plasma is used and 24 hours may be needed for a result. Some strains are slide-test positive. Results of both tests may show strain variation depending on whether the strains originate from pigs or cattle; cattle strains are significantly less reactive[22] *Staph. hyicus* may be isolated from a variety of farm animals but appears to be a significant pathogen only of pigs.

Staph. hyicus is incompletely inhibited on Baird-Parker agar. Colonies are initially greyish-blue and become brown or black after 48 hours incubation. Colonies on egg-yolk agar are surrounded by zones of incomplete clearing and there is no surface or sub-surface precipitation in the area immediately adjacent to the colony.

A high proportion of *Staph. hyicus* strains possess receptors for immunoglobulin G and will test positive for protein A, thus increasing the possibility of misidentification as *Staph. aureus*. In common with *Staph. hyicus* coagulase-positive tests, *Staph. hyicus* protein A-positive tests are much less likely to occur with strains from cattle.[22]

Staph. hyicus is non-pigmented and non-haemolytic on sheep-blood agar but small zones of lysis are usually seen if it is grown on heated (chocolate) blood agar. A CAMP-like factor causes zones of complete lysis on sheep-blood agar when the test strain is cultured with a strain of *Staph. aureus* that produces β-haemolysin. The characteristics of *Staph. hyicus* are discussed by Lämmler.[22]

Caution should always be exercised over results that presumptively identify a strain as *Staph. hyicus* because strains of a *Staph. epidermidis* subspecies isolated from chickens and bovine udders have been shown by Devriese and Oeding[24] to produce a heat-resistant nuclease and react in the tube coagulase test. These strains differ from *Staph. hyicus* in having no apparent pathogenicity for pigs and appear to occupy an intermediate species position. They were reported to be frequent inhabitants of poultry skin which has obvious implications for interpretation of coagulase tests if their occurrence is widespread.

Staph. intermedius also is coagulase-positive, but as it is not normally associated with food animals or humans its presence in foods is unlikely. Probably its major importance is that it may be routinely identified as *Staph. aureus* because it shares some characteristics with this species which are generally regarded as typical for *Staph. aureus* only.

Some other tests for identification of *Staph. aureus* are discussed by Devriese and Hajek.[25] Results of key tests to differentiate the coagulase-positive species are shown in Table 7. A study to establish the minimum number of tests needed for this purpose was carried out by Robertson[26] and resulted in the recommendation that tests for β-galactosidase and growth on P-agar[27] supplemented with acriflavin[28] should be used.

There is little or no requirement for extended testing to prove strains as coagulase-negative, emphasis necessarily being put on proving whether an isolate is coagulase-positive with the attendant risk that it may be enterotoxigenic. However, coagulase-negative strains that produce toxin have been reported and if similar strains are isolated additional proof that they are definitely coagulase-negative may be desirable. Unfortunately there appear not to be characteristics, apart from the lack of coagulase activity, that easily and reliably identify these organisms as a group. Probably the most satisfactory procedure, apart from investigating the presence of appropriate genes, is to determine the biochemical characteristics of a strain to name the species as one known not to produce coagulase. However, such a high level of precision is rarely necessary. If there is a suspicion that an apparently coagulase-negative strain is enterotoxigenic then tests for toxin production should be performed even though the species has not been determined.

Tests for phosphatase production using solid[28,29] and liquid media[30] may have a place because a much smaller proportion of coagulase-negative strains are phosphatase-positive. The phosphatase test is briefly described on page 27.

Jay[31] observed a considerable difference between *Staph. aureus* and coagulase-negative strains in their susceptibility to sodium borate. As with phosphatase testing, the distinction is not complete and it must be concluded that both tests are probably more appropriately applied as screening tests for *Staph. aureus*.

In summary, *Staph. aureus*, the species of *Staphylococcus* most important in food microbiology, cannot be identified with certainty on the evidence of only one characteristic. The importance especially of *Staph. hyicus*, but also of *Staph. intermedius*, is restricted to the complication they can add to identification of *Staph. aureus*. However, there are simple differentiating features[24] which can be added to the evidence of the coagulase test but their recognition is dependent on

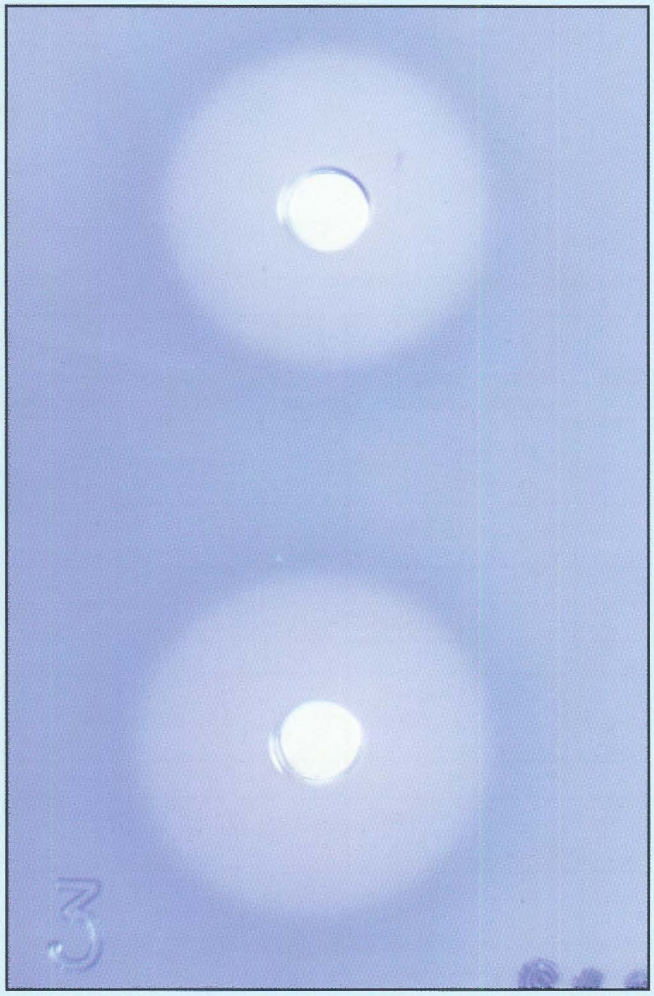
subculturing on sheep-blood agar and preferably also on DNAse agar.

(i) A positive clumping factor (slide coagulase) test on a pigmented culture is a characteristic only of *Staph. aureus.*

(ii) A pigmented strain which is also strongly DNAse-positive identifies the strain as *Staph. aureus.*

(iii) β-haemolysis surrounding a pigmented colony on sheep-blood agar also identifies the strain as *Staph. aureus.*

If only one of these characteristics is determined and the result is negative, then it is essential that at least one other is also examined. An acetoin test[32] also may be done; positive results are almost entirely limited to *Staph. aureus.*



Positive tests for thermonuclease indicated by halos of colour change from blue to pinkish-blue. The halos surround the test samples contained in wells cut in toluidine-blue agar. Photograph: A.H. Varnam and M.G. Evans. *Foodborne Pathogens: An Illustrated text.* Manson Publishing 1996.

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Table 8 – Differentiation of *Micrococcus* and *Staphylococcus* species.

Test	Micrococcus	Staphylococcus
Anaerobic fermentation of glucose[a]	–	+
Susceptibility to lysostaphin[b]	+	–
Susceptibility to furazolidone (100 µg)[c]	–	+
Susceptibility to bacitracin (0.04 units)[d]	+	–
Acid produced from glycerol aerobically in the presence of erythromycin 0.4 µg/ml[e]	–	+
Modified oxidase test.[f] It is essential that this test is done only with oxidase reagent made specially for differentiating staphylococci and micrococci	+	–

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Table 9 – Major Identifying Characteristics of Staphylococci Associated with Domestic Food Animals.

Species of Staphylococcus	Animal type	Colony pigment	Haemolysin	Coagulase	Clumping factor	Thermo-nuclease	Alkaline phos-phatase	Urease	Nitrate reduction	Aesculin hydrolysis
Staph. aureus	Cattle Poultry Sheep	+	+	+	+	+	–	–	–	
Staph. equorum	Horse Cattle	–	+* (slow)	–	–	–	+ (slow)	+	+*	+*
Staph. arlettae	Poultry	+	–	–	–	–	+ (slow)	–	–	–
Staph. gallinarum	Poultry	+*	+* (slow)	–	–	–	+ (slow)	–	+	–
Staph. intermedius	Poultry	–	+*	+	+*	+	+	–	+	–
Staph. hyicus	Pigs Poultry Cattle	–	–	+*	–	+	+	–	+	–
Staph. sciuri	Poultry	+*	–	–	–	–	+	–	+	+
Staph. caprae	Goat Horse	–	+* (slow)	–	–	–	+ (slow)	–	+	–

*10% or more of strains are negative.

Source: Kloos, W. (1997) in: Taxonomy and Systematics of staphylococci indigenous to Humans. Chapter 5 of *The Staphylococci in Human Disease.* Crossley, K.B. and Archer, G. L. (eds). Churchill Livingstone, New York.

DNAse Agar

DNAse Agar

Code: CM321

For the detection of microbial deoxyribonuclease enzymes, particularly from staphylococci.

Formula

	grams/litre
Tryptose	20.0
Deoxyribonucleic acid	2.0
Sodium chloride	5.0
Agar	12.0

pH 7.3 ± 0.2

Directions

Suspend 39 g in 1 litre of distilled water and bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

Description

Weckman and Catlin[1] suggested that DNAse activity could be used to identify pathogenic staphylococci after they had established a close correlation with coagulase production. Jeffries *et al.*[2] incorporated DNA in the agar medium to provide a simple method of detecting DNAse activity. Organisms are streaked on to the surface of the agar medium and incubated. The growth on the surface of the agar is then flooded with 1N hydrochloric acid. Polymerised DNA precipitates in the presence of 1N HCl and makes the medium opaque. If the organisms produce DNAse enzymes, in sufficient quantity to hydrolyse the DNA, then clear zones are seen around the colonies.

Good correlation was shown between DNAse production and coagulase activity when testing *Staphylococcus aureus* strains from clinical samples.[2,3,4] Both *Staph. aureus* and *Staph. epidermis* produce extracellular DNAse[5,6,7] but *Staph. aureus* produces greater quantities.[1,7]

A modification of the medium is to add mannitol (1% w/v) and phenol red or bromothymol blue (0.0025% w/v) as an indicator of mannitol fermentation.[8] The pH reaction around the colonies must be read before the plate is flooded with acid.

The DNAse reaction helps in the differentiation and identification of non-pigmented *Serratia marcescens*[9] (positive DNAse reaction) from *Klebsiella-Enterobacter* (negative DNAse reaction).

Normal HCl is bactericidal and the organisms cannot be recovered from the surface of the agar after flooding. The incorporation of dyes into the medium which can distinguish hydrolysis of DNA is a further modification which avoids the use of acid. Toluidine blue[8] and methyl green[10] form coloured complexes with polymerised DNA; these colours change as the DNA is hydrolysed.

It should be noted that toluidine blue can inhibit Gram-positive organisms and it is recommended that it is used to detect DNAse production by the Enterobacteriaceae.

Modifications to methodology and strict control of dye toxicity have however made it possible for toluidine blue to be used for working with staphylococci.

Technique

Inoculate the plates by spotting the organism onto the surface of the agar so that a thick plaque of growth is evident after 18 hours incubation.

Examine plates for colour changes in or around the colonies if mannitol/indicator or dyes have been added to the medium. In the absence of dyes, flood the plates with 1N HCl and allow them to stand on the bench (lids uppermost) for a few minutes. Look for zones of clearing around the colonies.

Appearance of colonies with media modifications

1 Mannitol/pH indicator:	
Yellow, with yellow zones	Mannitol +
Same colour as medium	Mannitol –
2 Toluidine blue:	
Pink zones in blue medium	DNAse +
No zones	DNAse –
3 Methyl green:	
Almost colourless zones	DNAse +
No zones	DNAse –
4 Acid flood:	
Well defined clear zones	DNAse +
No clear zones	DNAse –

Storage Conditions and Shelf Life

Store the dehydrated medium below 25°C and use before the expiry date on the label.

Store the prepared plates of medium at 2–8°C.

Quality Control

Positive control:
Staphylococcus aureus ATCC® 25923
Serratia marcescens ATCC® 8100

Negative control:
Staph. epidermidis ATCC® 12228
Klebsiella pneumoniae ATCC® 13883

References

References are shown on the following page.

Precautions

The DNAse reaction for staphylococci is an indication of pathogenicity. It cannot be used as the sole criterion for identification.

Small zones of clearing may be caused by other enzymes or organic acid production.[7]

Once the hydrochloric acid has been applied to the medium the plate must be read within a few minutes and further testing cannot be carried out by reincubation.

The methyl green must be purified by extraction with chloroform.[10] Toluidine blue varies in performance according to source.

Merck Toluidine blue 1273 is satisfactory for work with Gram-negative organisms. Note that this dye cannot be used for Gram-positive organisms.

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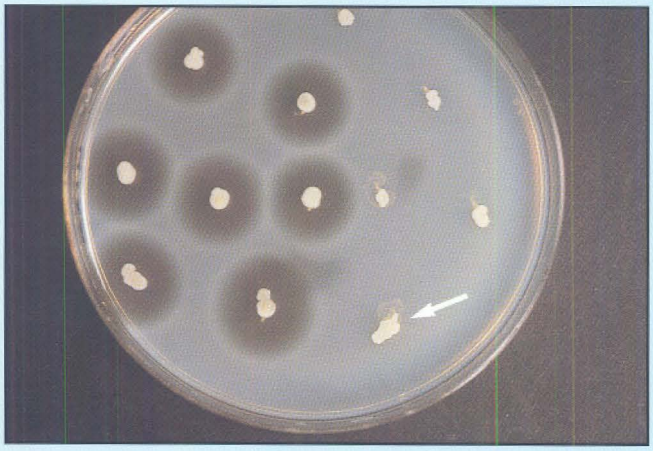

Typical appearance of a positive *Staph. aureus* DNAse test indicated by clear zones surrounding colonies on a DNAse agar plate flooded with hydrochloric acid. Colonies of *Staph. epidermidis* (arrowed) lack DNAse activity.

Staphylococcal Enterotoxins and Conditions for their Production

Staphylococcal food poisoning was first reported in 1884 in patients who had eaten cheese. Microscopic examination of the cheese showed clumps of cocci. Another outbreak in 1914 was associated with drinking milk from a cow with mastitis. Milk and milk products remain to this day a potential source of food poisoning which occurs following ingestion of enterotoxin produced during growth of *Staphylococcus aureus* in the food. Like botulism, staphylococcal food poisoning is an intoxication: it is not dependent on intestinal infection.

Ingestion of enterotoxin is quickly followed by onset of cramping, abdominal pain, nausea, vomiting and diarrhoea. Severely-effected patients may require rehydration therapy to compensate for fluid lost in diarrhoea and vomit. The condition generally is not life-threatening although a mortality rate of 5% has been reported. The severity of illness varies considerably in individuals but is always unpleasant and debilitating. As with other causes of food poisoning, the total economic effects of staphylococci intoxication are considerable.

For many years it had been supposed that *Staphylococcus aureus* alone amongst staphylococci was associated with enterotoxin production and consequent food poisoning. Coagulase production by food isolates was taken as presumptive identification of *Staph. aureus* and, accordingly, that they were potentially pathogenic but in general coagulase-negative strains have been considered insignificant. Thermonuclease production has also commonly been linked with *Staph. aureus* and detection of thermonuclease in foods from which viable organisms cannot be recovered following processing, has generally been regarded as showing that this species has grown in the food and enterotoxin may be present as well. However, with the introduction of more discriminatory techniques it has been possible to name other coagulase-positive, thermonuclease-positive species, that are enterotoxic, e.g. *Staph. intermedius.*

Strains of newly recognised coagulase-negative species are increasingly seen to be capable of producing thermonuclease and/or enterotoxin. Consequently, the emphasis put on coagulase-positive strains in food examination may lead to under-estimating the enterotoxigenic potential of other staphylococci present. Species known to produce coagulase, nuclease and/or enterotoxin are listed in Table 10. Note that not all species produce nuclease that is heat-stable (thermonuclease).

Enterotoxin is one of a range of extracellular products which are important in the pathogenesis of *Staph. aureus.* These include haemolysin, lipase, coagulase and nuclease. Approximately one-third of strains are able to produce enterotoxin and if conditions allow them to grow profusely, sufficient quantities of toxin to cause food poisoning may be formed in the food. Ingestion of quantities in the order of 300–500 nanogram is required. Because staphylococcal enterotoxins are antigenic, patients who have previously been exposed to them may be partially immune and show relatively mild symptoms or even remain asymptomatic. The enterotoxins are superantigens which stimulate monocytes and macrophages to produce cytokines. This immunological activity suggests that Staph. enterotoxins may also be involved in non-enteric disease states.

There are a number of immunologically distinct staphylococcal enterotoxins. They are termed enterotoxins A, B, C, D and E (SEA to SEE). SEC is divided into two sub-types. A further toxin previously designated enterotoxin F but now as TSST, is very similar to the enterotoxins but is responsible for toxic shock syndrome and not enteritis. δ-haemolysin produced by *Staph. aureus* is a surfactant and is also able to activate adenylate cyclase resulting in cyclic – AMP production and consequent diarrhoea similar to that produced by cholera toxin. This activity of the haemolysin may play a part in the diarrhoea seen in toxic shock syndrome and food poisoning.

Individual strains of *Staph. aureus* may produce multiple toxins. The most common patterns of production have been found to be enterotoxin C alone and enterotoxin C with TSST.[2] Where single toxins are detected SEA is most frequently found, followed by SED. SEE is least frequently found. Enterotoxin B is involved in staphylococcal enterocolitis, a rare but often fatal complication of abdominal surgery, but in the case of this condition there is not an association with pre-formed toxin in food. The enterotoxins of *Staphylococcus aureus* act directly on the gut and, from there via nerve pathways, on the area of the brain responsible for vomiting. This mode of activity in producing enteritis has led to these toxins being regarded as neurotoxins and appears unique. The enterotoxins are single polypeptide chains having molecular weights of 27000 to 35000. Lysine, tyrosine, aspartic acid and glutamic acid are the most abundant amino acids. Staph. enterotoxins are not destroyed by high cooking temperatures or proteolytic enzymes. It is their resistance to heat and to proteolysis in the gut which makes it important that foods in which *Staph. aureus* may have multiplied during manufacture should be tested for toxin following heat processing and consequent cell death.

The ability of *Staph. aureus* to grow and produce detectable amounts of enterotoxin is dependent on the nature of the food product, what it is made of and whether it is raw, processed in some way, or fermented, and on the interactive effect of the differing conditions that exist during the periods of handling before and after processing and during storage. *Staph. aureus* can be expected in products of animal origin and those that have been handled during manufacture. The incidence of the different enterotoxins produced by strains recovered from various sources varies considerably. SEA is produced by strains isolated from humans but the incidence appears to vary widely with geographical location of food poisoning outbreaks. Variation also occurs amongst *Staph. aureus* isolates from different foods. SED producers have been found amongst poultry strains. SEA and SED-producing strains have been found amongst isolates from sheep and SEA, SEB and SEC strains were detected in goat milk. However, it it difficult to establish the real incidence of the different toxins produced as reports in the literature are sometimes contradictory.

Attempts to associate enterotoxin production with other properties such as gelatinase, phosphatase, lecithinase and deoxyribonuclease production, or with fermentation of various carbohydrates, have shown little difference between enterotoxigenic and non-enterotoxigenic coagulase-positive strains.

Nutrition availability has a considerable effect on enterotoxin production. Easily metabolisable sources of carbon have been shown to influence toxin production but not in every case to increase it. The production of the different toxins varies with the nature of carbohydrates; glucose and glycerol have actually been shown to repress toxin production under defined conditions.[1] Similarly contradictory behaviour occurs with some amino acids including glutamate, proline, histidine, aspartate, alanine and glycine, but the mechanisms appear not to be similar to those associated with the use of glucose as a source of energy.[2]

A single cell of *Staph. aureus* can theoretically initiate growth and enterotoxin production in a suitable food. However, in general, *Staph. aureus* is poorly competitive and multiplication can be inhibited in the presence of coliforms, *Pseudomonas spp.* and lactobacilli. Some microbial associations allow adequate growth of *Staph. aureus* but enterotoxin production may be suppressed.

Growth of enterotoxigenic and non-enterotoxigenic strains

together may allow production of enterotoxin to occur even when the non-toxigenic strain is present in much greater numbers.[3]

Atmospheric composition influences both growth and enterotoxin production but the specific effect varies with the type of toxin. Toxin may be produced under anaerobic conditions but greater amounts are produced when oxygen is present.

Temperature has a considerable effect on growth and consequent toxin production. Foods stored at temperatures above 10°C must constitute a risk of food poisoning.

The optimum pH for growth of *Staph. aureus* is 6.0 to 7.0 but the actual range of pH over which it can grow is 4.0 to 9.8 when other cultural influences are optimal. However, foods having pH values below 5.0 appear not to support enterotoxin production. Optimal pH values differ for the various enterotoxins and the acidulant used to adjust pH of a food may determine whether toxin is produced.

Staph. aureus is able to grow over a much wider range of water activity than other foodborne pathogens and the aw requirements for growth and toxin production are much the same. Associated with the organism's ability to thrive in decreased water activity is a tolerance of high levels of sodium chloride. Salt-preserved foods can therefore be at risk. High salt concentrations are commonly used in selective culture of staphylococci. The temperature limits for growth of *Staph. aureus* may be widened in the presence of raised concentrations of salt but toxin production may be influenced adversely.

As a rule, oxygen tension and competition from other microorganisms have a greater effect on enterotoxin production than temperature, pH and water activity.

A summary of the conditions necessary for growth of *Staph. aureus* and enterotoxin production is given in Table 11.

References

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Table 10 – Species and Subspecies of *Staphylococcus* that Produce Coagulase, Nuclease and/or Enterotoxins.

Species or subspecies	Coagulase	Nuclease	Enterotoxin
S. aureus	+	TS	–
subsp. *anaerobius*		TS	–
subsp. *aureus*	+	TS	+
S. intermedius	+	TS	+
S. hyicus	(+)	TS	+
S. delphini	+	–	
S. schleiferi			
subsp. *coagulans*	+	TS	
subsp. *schleiferi*	–	TS	
S. *caprae*	–	TL	+
S. *chromogenes*	–	w	+
S. *cohnii*	–	–	+
S. *epidermidis*	–	–	+
S. *haemolyticus*	–	TL	+
S. *lentus*	–		+
S. *saprophyticus*	–	–	+
S. *sciuri*	–		+
S. *simulans*	–	v	
S. *warneri*	–	TL	+
S. *xylosus*	–	–	+

Key: + = positive
(+) = weakly positive
– = negative
w = negative or weakly positive
v = variable
TS = heat stable
TL = heat sensitive

Source: Modified from Jay, J.M. *Modern Food Microbiology* 4th edition. Van Nostrand Reinhold, New York, 1992.

Table 11 – Influences on Growth and Enterotoxin Production by *Staphylococcus aureus.*

Influence	Growth		Toxin Production	
	Optimum	Range	Optimum	Range
Temperature, °C	37	4–48	35–40	10–45
pH	6.0–7.0	4.0–9.8	5.3–7.0	4.8–9.0
Eh, mV	> +200	< –200	> +200 to > +200	Not determined
aw.	0.98≥0.99	0.83≥0.99	≥0.90	0.86≥0.99
Atmosphere	Aerobic	Aerobic to anaerobic	5.0–20% O_2	Anaerobic to aerobic
Sodium chloride %	0.5–4.0	0–20	0.5	0–20

Table adapted from Adams, M.R. and Moss, M.O. (1995) *Food Microbiology*, Table 7.7 Royal Society of Chemistry, Cambridge, UK.

Tests for Staphylococcal enterotoxin

The recognition by Dack[1] that an outbreak of food poisoning had been caused by a pigmented haemolytic staphylococcus was quickly followed by the development of tests to confirm the role of enterotoxin in similar cases. Early animal studies showed the usefulness of monkeys in routine detection of enterotoxin when Jordan and Broom[2] induced vomiting in these animals by feeding preparations containing enterotoxin. This removed the need to use human volunteers, the only investigative method Dack had available. Vomiting is rare in monkeys and is considered to be acceptable proof of a positive test if the test sample induces this reaction in at least two animals. Further developments led to the introduction by Bergdoll[3] of the monkey feeding test in which food samples suspected to contain enterotoxin are administered directly to the stomach. The use of primates in staphylococcal enterotoxin research was extended by Wilson[4] who orally administered partially purified preparations of enterotoxins to chimpanzees.

There are practical and cost difficulties in the use of monkeys. The search for alternative species showed unacceptably low susceptibility of dogs,[5] pigs[6] and rabbits.[7] Kittens were found to be a suitably responsive animal and their use was first described in 1936.[5] These animals are not very sensitive to enterotoxin when it is administered by mouth and the intraperitoneal or intravenous routes are used. Tests may also be carried out on adult cats.[8]

A skin test in guinea pigs which removes the need for oral or parenteral administration was described[9] but did not enter general use.

Cats and monkeys have for many years been the usual test animals and attempts to replace the use of mammals with lower animals including frogs, nematodes and fish have been unsuccessful.

Detection of staphylococcal enterotoxins by their effect on cells and cultured tissue is less useful than for other bacterial toxins, but binding of A, B and E enterotoxins to mouse spleen cells[10] and the effect of enterotoxin A on human peripheral lymphocytes[11] have been employed.

A test system which exploits the superantigen nature of enterotoxin A has been described as an alternative to animal and ELISA immunological tests for direct detection of toxin in foods.[12] ELISA tests do not detect functional activity of enterotoxin and this can cause problems with heat-processed foods in which toxin may still be biologically active but no longer serologically active. Mitogenicity as a marker for active enterotoxin A had been suggested earlier[13] and the alternative detection system[12] employs demonstration of the proliferation of human or rat T-lymphocytes in the presence of enterotoxin.

Immunological test systems are firmly established for routine use. The standard microslide Ouchterlony gel diffusion technique has now largely been replaced by ELISA and latex agglutination methods. In 1946 Oudin[14] had shown that precipitation occurs in an agar gel at a position where antigen travelling under the effect of gravity downwards through the gel meets the corresponding immobilised antibody in optimal proportion. Following description of this single gel diffusion method a double-diffusion method was devised by Oakley and Fulthorpe.[15] In this procedure both antigen and antibody travel through the gel, their concentrations decreasing the further they travel. When they meet, precipitation occurs at a position dependent on the relative concentrations of the reactants. Ouchterlony[16] utilised agar plates for this double-diffusion test. The Ouchterlony test system was later miniaturised so that it may be carried out on glass slides to economise on reagents. This microslide technique has in turn been modified specifically for the analysis of staphylococcal enterotoxins.[17,18] A miniaturised tube method for gel diffusion tests has also been developed.[19]

Gel diffusion technology is slow and technically-demanding and now ELISA and latex agglutination are preferred for routine use.

Competitive and sandwich ELISA procedures have been applied to the detection of staphylococcal enterotoxin but the sandwich procedure has been adopted more widely. The early ELISA assays used polyclonal antibodies but now monoclonal antibodies are more usual. Most test methods employ microtitre plates.

Reversed Passive Latex agglutination (RPLA) has largely replaced haemagglutination and haemagglutination-inhibition methods since Saloman and Tew[20] showed that antibody to enterotoxin can be bound to particles of latex. Test systems that have been developed using passive agglutination of latex are similar to the reversed passive haemagglutination technique described by Silverman,[21] only the antibody carrier being different.

The Oxoid Staphylococcal enterotoxin kit (SET-RPLA) is pictured on page 45.

Other immunological techniques have been applied to staphylococcal enterotoxin:

Immunofluorescence methodology has been used to detect cell-associated enterotoxin B[22], enterotoxin B in culture media[23] and in foods.[24] The methods appear not to have been adopted to any extent.

Radioimmunoassay has been employed as an alternative to fluorescence assay,[25,26] employing radioactive iodine as the label. This procedure also has not become widely used for enterotoxin detection.

Other procedures that have been used include electrophoresis,[27] electroimmuno-diffusion[28] reversed immunoosmophoresis[29] and affinity chromatography.[30] Their use appears not to have extended beyond research applications.

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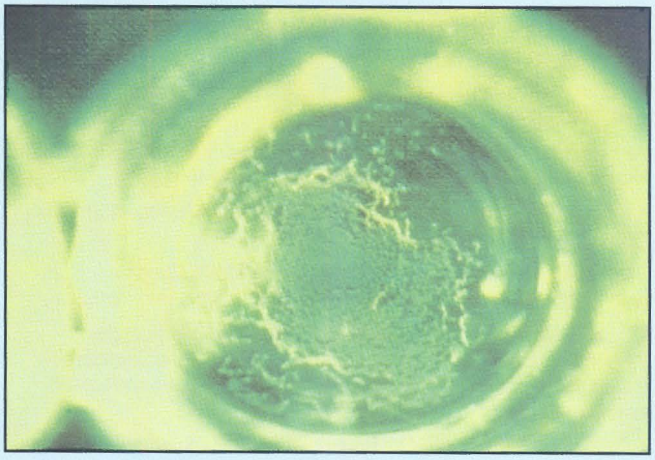
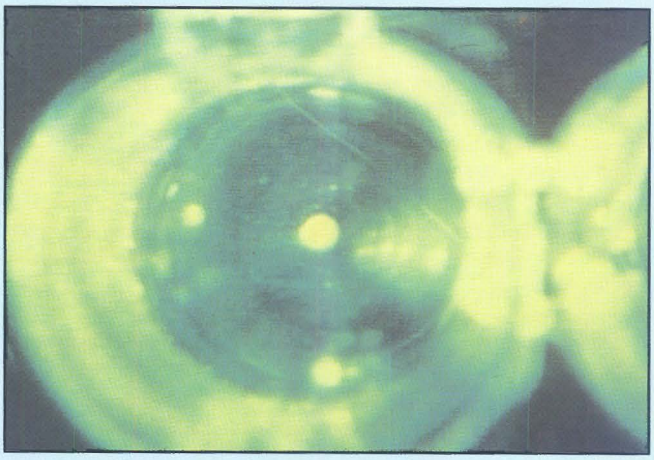
Staphylococcal RPLA Kit

Code: TD900





Testing for Staphylococcal enterotoxin using the Oxoid SET-RPLA kit. (Reversed Positive Latex Agglutination). Positive top, Negative bottom.
Oxoid Marketing collection.

Direct testing for toxin in foods can identify the specific cause of food poisoning when it has occurred.

Toxin-producing strains of *Staph. aureus* isolated in culture from foods and the food preparation environment can be identified by their specific toxin type and consequently linked to samples of food that are suspected of causing food poisoning.

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