AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

ANIMAL HEALTH COMMITTEE

SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Bovine Mastitis

Bacteriology

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1. Introduction

Mastitis is an inflammatory change of the mammary gland which, along with physical, chemical and microbiological changes, is characterised by an increase in somatic cells, especially leucocytes, in the milk and by pathological changes in the mammary tissue.

2. Definitions

International Dairy Federation (1987).

2.1. Normal Udders

Normal udders are those which show no outward signs of a pathological condition and the milk from which is free from pathogenic organisms and has a normal cell count (<500 000/mL).

2.2. Latent Infections

Latent infections are present when the milk shows the presence of pathogenic organisms, but nevertheless has a normal cell count.

2.3. Subclinical Mastitis

Subclinical mastitis shows no macroscopic evidence of inflammation, but examination of the milk reveals udder infection, an increased cell count and also alterations in the chemical properties of the milk.

2.4. Clinical Mastitis

2.4.1. Acute Mastitis

Acute mastitis is present when there are obvious symptoms of inflammation of the udder such as heat, pain and swelling. The milk is macroscopically abnormal and the animal may have a high temperature.

2.4.2. Subacute Mastitis

Subacute mastitisis present when there are no obvious changes in the udder but when there are persistent clots especially in the foremilk.

2.5. Non-Specific or Aseptic Mastitis

Non-specific or aseptic mastitis is present when there is no recognisable infection and the symptoms may be subclinical or clinical.

2.6. Chronic Mastitis

Chronic mastitis occurs when a quarter fails to respond to treatment over time. The quarter may atrophy or show abnormal clinical changes for the rest of the animal's life.

3. Mastitis Pathogens

Staphylococci and streptococci are the main organisms responsible for mastitis in cattle. The numerous other organisms which may be isolated from cases of mastitis play a relatively minor role. With the increasing use of intramammary antibiotics and the replacement of hand milking with mechanical milking methods, the incidence of streptococcal infections has declined whereas the incidence of coagulase positive staphylococci has increased.

More recently, pathogens originating from the cow's environment, in particular *Escherichia coli* and *Streptococcus aberis* are becoming important causes of clinical mastitis.

4. Conditions for Infection

Infection of the mammary gland usually takes place via the teat duct and apart from trauma is dependent on certain factors as follows.

(a) On the presence, concentration, pathogenicity and virulence of the microorganisms in the environment.
(b) On the condition and characteristics of the teat orifice and the streak canal.
(c) On the immunological and cytological status and the reaction of the mammary gland. Injuries to teat orifice and duct must also be considered.

During mechanical milking the following factors are important in relation to the initial entry of infection:

(a) traumatic damage to the mammary gland and teat and particularly to the teat orifice;
(b) mechanical transmission of pathogenic microorganisms from one animal to another;
(c) possible penetration of pathogenic microorganisms through the streak canal during milking .

5. Collection of Milk Samples

Prepare the cow carefully for sampling. Wash the teats with running water, or water containing a skin disinfectant such as Cetrimide. Try to avoid wetting the udder because it will be difficult preventing any excess water from contaminating the milk sample during collection. Dry with an individual soft cloth, paper towel and wipe teat orifice firmly with a cotton or gauze pledget soaked in 70% ethanol (C_2H_5OH), a separate pledget being used for each teat. The sides of the teat should be dried with the same wad after squeezing dry. The teats on the side of the udder opposite to the operator should be cleaned first. To reduce contamination from the streak canal it is best to discard the first squirt of milk.

The sample should be taken into a sterile container with a screw cap; 15–20 mL of milk being collected from each quarter. Samples should be collected from the teats nearest the operator first.

All samples should be clearly labelled or coded with the identity of the cow, the quarter, and the date of collection.

When collecting the sample, hold the container with the open end well above the hand in order to prevent any sprayed milk from running off the collector's hand into the container. Squirt the milk from the teat into the container which is held at a 45° angle. When sampling, the teat end should not touch the container.

Collect the sample with minimal pressure. If milk let down has not occurred during preparation do not 'strip' the teat over the open vial, otherwise skin debris or dirt is added to the sample.

The hands of the person taking the samples should be washed between each cow in a solution of disinfectant.

5.1. Handling and Storage of Samples

Following collection, all screw caps should be tightened down, bottles should be placed in a carrying rack and samples should be chilled and forwarded to the laboratory in an esky containing freezer bricks. Dispatch the samples to the laboratory as soon as possible after sampling.

On arrival at the laboratory, the samples should be cultured immediately or held at 4–5°C. For best results, bacteriological examination should be commenced within 24 hours of collection.

6. Culture Media

Dried media constituents should be used as far as possible and the media prepared according to the manufacturer's directions.

It is recommended that blood agar (BA) be used for primary examinations.

Either bovine (preferably calf) or ovine blood may be used for preparation of blood agar. Staphylococcal antihaemolysins present in the blood of certain animals may inhibit the α- and β-haemolysins of *Staph. aureus*. Therefore, each new lot of blood should be tested with a standard strain of *Staph. aureus* which produces a 4–6 mm wide zone of complete haemolysis (α-haemolysin) and a strain which produces a distinct zone of darkening around the growth (β-haemolysin).

Rabbit, horse or human bloods are not satisfactory for use in blood agar.

7. Culture Methods

7.1. Preparation of the Sample

In refrigerated samples, bacteria will be concentrated in the fat layer. Such samples should be warmed to 25°C (in a 37°C water bath for not more than 15 min) before mixing and plating.

Just before removing the inoculum for plating, invert gently several times.

7.2. Inoculation of Plates

Plating methods in general use for the microbiological diagnosis of bovine mastitis may not be strictly quantitative, and some variation is thus permissible as regards the volume of inoculum and surface area of medium over which the inoculum is spread. A standard 10 µL loop should be used for the inoculum. For special work an inoculum of 25 µL should be used as milk samples from infected quarters occasionally contain less than 200 bacteria/mL (two colonies or fewer/10 µL). Suitable combinations of inoculum size and surface area are a follows:

(a) 10 µL on 1/4 of a 10 cm plate;
(b) 25 µL on 1/3 or 1/2 of a 10 cm plate; or
(c) 50 µL on a whole 10 cm plate.

The inoculum should be spread so that at least some single, well isolated colonies will result.

7.3. Preincubation of Milk Samples

In special circumstances where only a small number of pathogenic microorganisms are likely to be present, it may be useful to preincubate the milk samples at 37°C for 6–18 hours. Only very carefully collected samples can be treated in this manner as contaminating organisms may overgrow the pathogens.

7.4. Incubation of Cultures

Cultures should be incubated at 37°C and examined after 18–24 hours for colonies of streptococci, staphylococci or other organisms and then reincubated for at least another 24 hours. When obviously abnormal milk consistently gives no growth following the recommended routine procedures it may be desirable to consider incubation at different temperatures or times either anaerobically or with added carbon dioxide. Under such circumstances examinations of stained milk or sediment smears may be valuable.

7.5. Selective Enrichment Media

The use of liquid selective enrichment media may be helpful in order to isolate special bacteria and this is particularly advisable if sampling conditions are difficult.

8. Bacteriological Diagnosis of Infection

The procedure adopted may vary according to the reason for examination of the milk samples (Francis, 1985). In general, the samples submitted can be divided as follows.

(a) Clinical samples, or samples from non-responsive quarters.
(b) Herd investigations — samples from non-responsive problem herds. These will be primarily subclinical cases.
(c) Samples from individual cows for examination for normality (e.g. house cows).

The examinations should be carried out as in Table 1.

In the diagnostic laboratory bacteriological procedures should be kept as simple as possible

Table 1. Selection procedures used to examine milk according to reason for the investigation

Somatic cell count rapid mastitis test	*Examination of stained smears*	*Bacteriological examination*	*Test for inhibitory substances*
		(a) Clinical samples	
No	Yes	25 or 50 µL inoculum	Yes if no growth
		(b) Herd investigations	
Yes	No	10 µL inoculum	Only if recent antibiotic therapy
		(c) Normality check	
Yes	No	25 or 50 µL inoculum	No

(Fig. 1). To this end it is recommended that primary isolation be attempted by direct culture onto thin BA plates. Very thin plates (or a thin overlay of BA on nutrient agar) are necessary to correctly determine the haemolytic patterns of streptococcal isolates. Plates should be examined at 18 hours and again at 42 hours.

Some organisms particularly *Pseudomonas* spp. which may at times be important are often difficult to isolate and the common pathogens may also be present in very small numbers in milk. It is, therefore, recommended that the milk samples also be incubated overnight at 37°C and again plated onto very thin BA plates. These plates should be examined at 18 hours (and 42 hours). For this examination to be worthwhile, care must be exercised in the collection of samples.

If there is no growth present on the plates the milk should be tested for inhibitory substances (see 11.6.).

If inhibitory substance cannot be detected, it may be necessary to examine the samples for the presence of other microorganisms such as anaerobes, mycoplasmas and fungi.

9. Identification of Mastitis Pathogens

See National Mastitis Council (1969).

9.1. Streptococci

Colonies on BA will be small, smooth, translucent, convex and may be surrounded by a zone of greenish discoloured erythrocytes (α-haemolysis), by a narrow or wide clear zone of lysed cells (β-haemolysis) or may be nonhaemolytic (γ colonies). Single colonies should be picked off and tested in the CAMP test. From the CAMP test plate single colonies should be picked off and tested for aesculin hydrolysis in aesculin broth and for inulin fermentation. The characteristics differentiating the principal species of mastitis streptococci are as follows (Table 2).

Table 2. Differential characteristics of the principal *Streptococcus* sp. causing mastitis

	Haemolysis	*Aesculin*	*CAMP*	*Inulin fermentation*
S. agalactiae	a, b or g	–	+	–
S. dysgalactiae	a or g	– or +[1]	–	–
S. uberis	a or g	+	– or +[2]	+

[1] About 30% positive; [2] About 30% positive.

Other streptococci may sometimes be associated with mastitis and would require further tests for identification.

The identification of *S. dysgalactiae* and *S. uberis* may need to be confirmed and the following set of tests has been recommended (Table 3).

9.2. Staphylococci

S. aureus produces creamy, greyish white or golden yellow haemolytic colonies on BA, 3–5 mm diameter in 18 hours.

9.2.1. α-Haemolysin

Clear zone of complete lysis, generally extending 2 mm or more from the edge of the colony.

9.2.2. β–α Lysin

Zone of darkened RBC.

9.2.3. δ–Toxin

Some strains may also produce narrow well defined zones of complete haemolysis due to this toxin.

Strains which produce α, or α–β lysins are usually coagulase positive. Colonies producing narrow haemolytic zones (1 mm or less) or partial haemolysis are generally coagulase negative staphylococci which may infect the udder and cause elevated cell counts in the milk.

Although the colony characteristics and the type of haemolysis on BA are usually sufficient to identify *S. aureus*, the following tests (Table 4) are recommended to differentiate between *S. aureus*, *S. epidermidis* and *Micrococcus* spp.

9.3. Coliform Bacteria

These may cause cases of non-clinical or clinical mastitis. *E. coli* and other coliform bacteria produce large, moist grey colonies on BA.

Table 3. Tests to confirm the identification of *Streptococcus* sp.

	S.. agalactiae	*S. dysgalactiae*	*S. uberis*
Catalase	–	–	–
Coccus morphology	+	+	+
CAMP reaction	+	–	+ or –
Aesculin fermentation	–	– or +	+
Inulin fermentation	–	–	+
Raffinose fermentation	–	–	–
Lancefield coupling	B	C	NA
Growth at 45°C	–	–	+

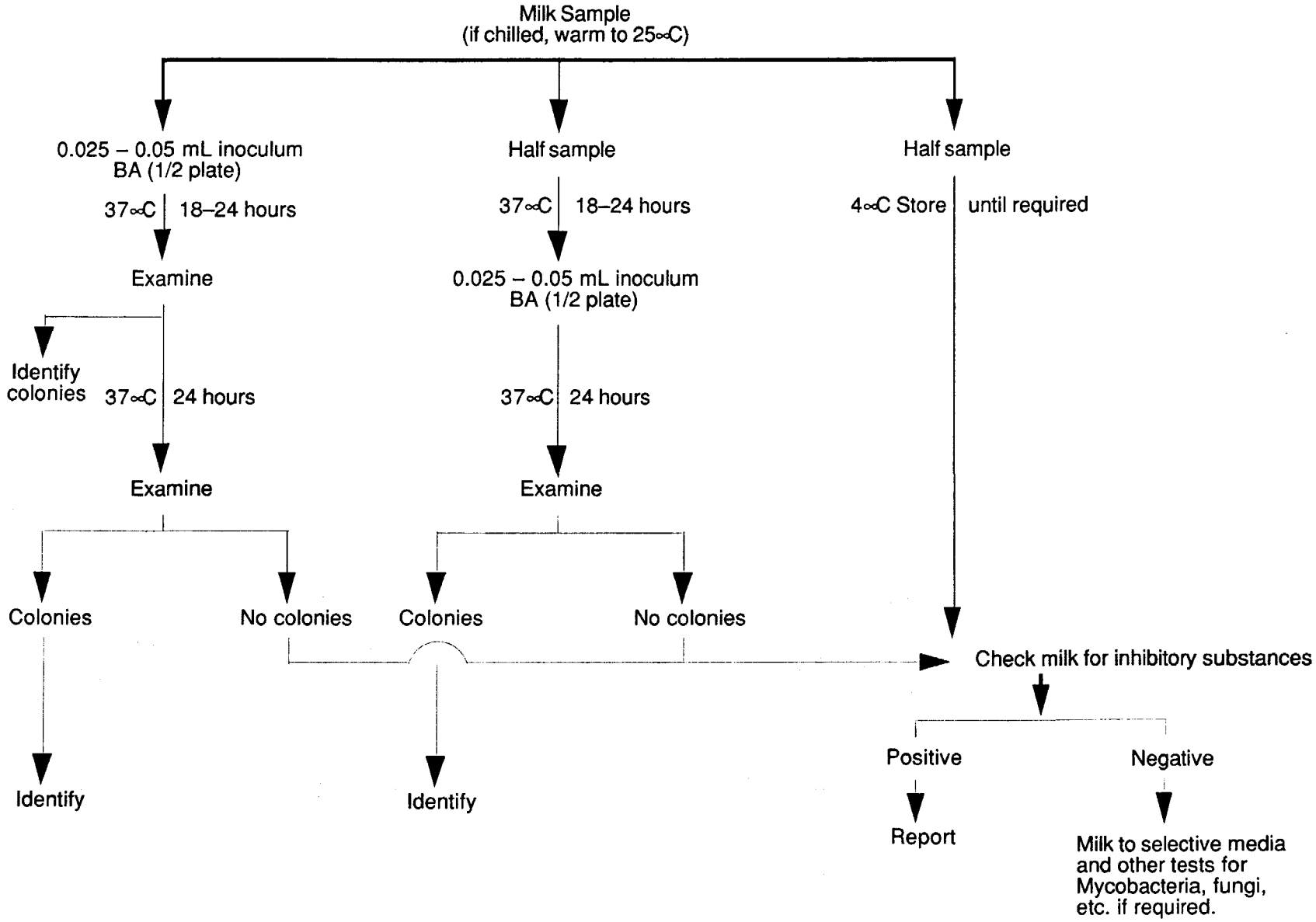

Figure 1. Examination of clinical milk samples

9.3.1. Confirmation on TSI Agar Slants

Inoculate TSI agar by streaking growth from the BA plate on slant and by stabbing butt of the agar. After 18-48 hours incubation at 37°C coliforms produce yellow colour on the slant (acid production) and yellow colour and gas bubbles in the butt of the medium. Further identification should employ standard tests.

9.4. Pseudomonads

These organisms may produce a severe form of mastitis which usually occurs sporadically but may become a serious herd problem. The colonies on BA are usually large, irregular and spreading, greyish in colour with dark centres and translucent edges. The production of a deep blue—green pigment (pyocyanin) is a distinctive characteristic of *Ps. aeruginosa*. This pigment produces a metallic sheen on surface growth and diffuses into adjacent media. The pigment is not produced by all strains. Many strains are haemolytic and most have a characteristic, strong, grape-like odour (trimethylamine).

The cyclic activity of pseudomonads in the bovine udder frequently causes difficulty in detecting their presence. Infection is best confirmed by repeated isolation on blood agar from successive milk samples. The culture of preincubated milk samples, which have been carefully collected under aseptic conditions, may be helpful in confirming infection due to *Pseudomonas* spp.

9.5. Actinomyces pyogenes and Corynebacteria

Actinomyces (Corynebacterium) pyogenes, *C. bovis* and *C. ulcerans* can infect the udder and cause inflammation.

A. pyogenes usually produces a very acute mastitis with grossly abnormal milk containing numerous Gram positive diptheroids.

C. bovis is often regarded as a harmless commensal. However, there is evidence that it may produce inflammation when found intramammarily and may be responsible for elevated leucocyte counts.

C. ulcerans is occasionally isolated from cases of mastitis. Only in very young cultures are typical diphtheroid rods seen and after 24–26 hours the cells become coccoid. The addition of 10% carbon dioxide greatly enhances the colony size and haemolytic zone.

Table 4. Tests to differentiate between *S. aureus*, *S. epidermidis* and *Micrococcus* spp.

	S. aureus	*S. epidermidis*	*Micrococcus*
Gram stain	Gm +ve cocci	Gm +ve cocci	Gm +ve cocci
Coagulase	+	–	–
Anaerobic fermentation of glucose	+	+	–
Anaerobic fermentation of mannitol	+	–	–

9.5.1. Colonies on Blood Agar

A. pyogenes — small (1.5–2 mm) circular convex opalescent colonies with smooth glistening surfaces, surrounded with a sharp haemolytic zone not more than 1 mm wide after 48 hours incubation.

C. bovis — colonies are seldom visible before 48 hours incubation. They are about 1 mm diameter and are usually found in the fatty areas of the streaked milk (require oleic acid). Usually not haemolytic, they are white to creamy, circular, opaque with entire edges and have a rough surface (when magnified).

C. ulcerans — after 24 hours at 37°C are circular, opaque, off-white in colour with a dull surface and later produce a zone of weak haemolysis. In six- to eight-hour nutrient broth cultures, the cells are non-branching irregular or club-shaped rods. In cultures incubated for 24–48 hours, coccobacillary forms predominate.

The differential characteristics of *A. pyogenes* and Corynebacteria can be summarised as shown in Table 5.

9.6. Bacillus cereus

This organism is widely distributed and is one of the most common of the aerobic spore forming bacteria found in the soil. It is generally considered a saprophyte but it can infect the udder, causing acute gangrenous mastitis. Death of the cow may result. Large numbers of organisms are present in the milk during the early stages of acute infection and can be demonstrated in smears of unincubated milk. However, few or no organisms may be found when the secretion is cultured with a standard inoculum 24 hours after the acute signs of disease develop.

The organisms are large, motile, Gram positive, spore forming rods which stain irregularly, have square to slightly rounded ends and form chains. Colonies on blood agar are haemolytic with a greenish grey colour and ground glass appearance. They may have a filamentous margin.

9.7. Other Microorganisms

Other microorganisms may occasionally be encountered although special media may be required for their isolation (Table 6).

Table 5. Differential characteristics of *Actinomyces pyogenes* and *Corynebacteria*

	C. bovis	*C. ulcerans*	*A. pyogenes*
Catalase	+	+	–
Requirement for Tween 80	+	+	+
Urease	+	+	–
Growth in 9% NaCl	+	–	–
Butterfat hydrolysis	+	–	+
Starch hydrolysis	–	+	+
Casein hydrolysis	–	–	+
Inhibition of Staph haemolysin	–	+	–

Table 6 Microorganisms isolated from mastitic udders requiring special culture media

Leptospira hardjo		
Mycobacterium bovis		
Mycobacterium fortuitum		
Mycobacterium lacticola		
Nocardia asteroides		
Nocardia caviae		
Mycoplasma spp.		
Fungi:	Yeasts	Moulds
	Cryptococcus	*Pichia farinosa*
	Candida	*Absidia ramosa*
	Trichosporon	*Absidia corymibifera*
	Geotrichum	*Aspergillus fumigatus*
Prototheca		

10. References

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11. Appendixes

11.1. Media

11.1.1. Blood Agar

Oxoid Blood Agar Base No. 2	40 g
Agar	15 g
Distilled water	1 L

Suspend BA base and agar in the distilled water, and bring to the boil to dissolve completely.
Mix and sterilise by autoclaving at 121°C for 15 min.
Cool to 50°C and add 7% sterile sheep or calf blood.

11.1.2. Aesculin Broth

Aesculin	1 g
Ferric citrate	0.5 g
Peptone water	1 L

Dissolve the aesculin and iron salt in the peptone water and sterilise at 115°C for 10 min.

11.1.3. Serum Water Sugars

Evans peptone	4 g
Disodium hydrogen phosphate, Na_2HPO_4	0. 8 mL
Distilled water	800 mL
Sterile bovine serum	150 mL
Bromocresol purple 0.2%	10 mL

Dissolve the peptone and phosphate in the water, steam at 100°C for 15 min and filter.

Adjust pH to 7.6–7.8 and add the indicator.

Sterilise at 115°C for 20 min. Allow to cool and add serum (sterilised by filtration).

Distribute into sterile bijou bottles in 2.7 mL amounts.

Add sterile carbohydrate to 1% concentration when required (0.3 mL of 10% solution).

11.1.4. Triple Sugar Iron Agar (TSI)

Oxoid TSI Agar	65 g
Distilled water	1 L

Suspend the TSI agar in the water and bring to the boil to dissolve completely.

Mix well and distribute 8 mL into McCartney bottles. Autoclave at 121°C for 15 min.

Allow the medium to set in sloped form with a butt about 125 mm long.

11.1.5. CAMP Test

Heavily inoculate a BA plate with two streaks of strain of *S. aureus* known to produce β–lysin at right angles to each other to form a cross. Diagonally across each quadrant so formed, a streak of a test streptococcal culture can be inoculated. The ends of the streak of streptococcal culture should just not come in contact with the staphylococcus streak.

Inoculate for 24 hours at 37°C and look for augmentation of haemolysis.

11.1.6. Tests for Inhibiting Substances

A number of procedures are available. Some are used in dairy technology to test milk for antibiotics. Two procedures often used in Australia are Naylor (1960) and Keogh (1961).

More sensitive tests are available commercially, in kit form. They are the Delvotest, distributed by Nightingale Chemicals Pty Ltd, and the STAR test distributed by CSL. They should be performed according to the manufacturer's instructions.