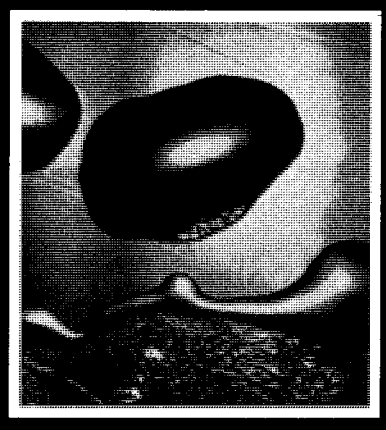

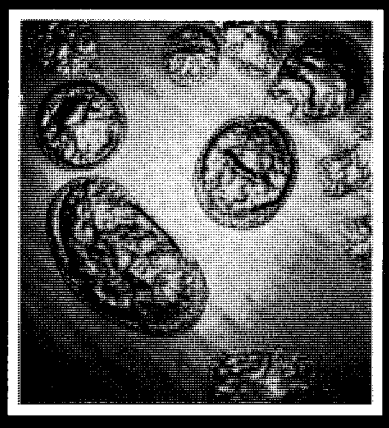

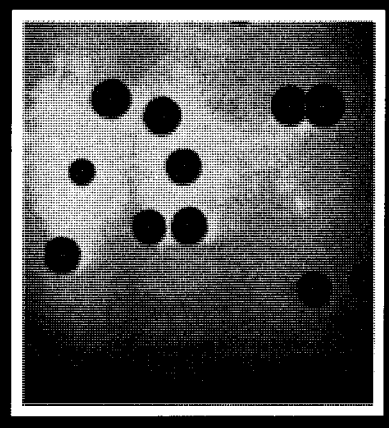

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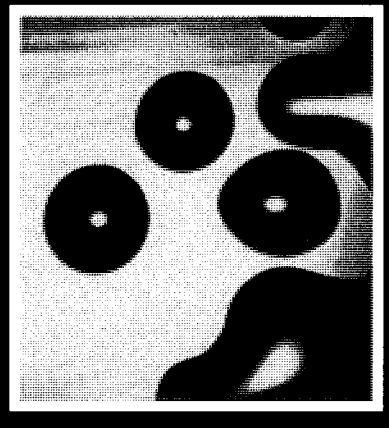

World Health Organization
Geneva

The World Health Organization was established in 1948 as a specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health. One of WHO's constitutional functions is to provide objective and reliable information and advice in the field of human health, a responsibility that it fulfils in part through its extensive programme of publications.

The Organization seeks through its publications to support national health strategies and address the most pressing public health concerns of populations around the world. To respond to the needs of Member States at all levels of development, WHO publishes practical manuals, handbooks and training material for specific categories of health workers; internationally applicable guidelines and standards; reviews and analyses of health policies, programmes and research; and state-of-the-art consensus reports that offer technical advice and recommendations for decision-makers. These books are closely tied to the Organization's priority activities, encompassing disease prevention and control, the development of equitable health systems based on primary health care, and health promotion for individuals and communities. Progress towards better health for all also demands the global dissemination and exchange of information that draws on the knowledge of all WHO's Member countries and the collaboration of world leaders in public health and the biomedical sciences.

To ensure the widest possible availability of authoritative information and guidance on health matters, WHO secures the broad international distribution of its publications and encourages their translation and adaptation. By helping to promote and protect health and prevent and control disease throughout the world, WHO's books contribute to achieving the Organization's principal objective — the attainment by all people of the highest possible level of health.

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Laboratory diagnosis of group A streptococcal infections

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Contents

Foreword

At the close of the twentieth century, infections with *Streptococcus pyogenes* (Lancefield group A) and their suppurative and non-suppurative sequelae are still important medical and public health problems throughout the world, whatever the climatic, ecological and cultural conditions may be.

Group A streptococcal infections of the upper respiratory tract are among the most common bacterial infections, especially in young children. Furthermore, in many countries undergoing industrialization, the non-suppurative sequelae of group A streptococcal infection, acute rheumatic fever and acute post-streptococcal glomerulonephritis, represent significant causes of morbidity and mortality. In these developing countries, where two-thirds of the world's population live, the difficulties in diagnosing and treating the infections are compounded by insufficient resources for rehabilitative cardiac surgery in complicated cases of rheumatic heart disease. The resurgence of rheumatic fever and severe systemic group A streptococcal infections, such as streptococcal toxic shock syndrome, erysipelas and necrotizing fasciitis, which have been reported from North America, Scandinavia, the United Kingdom and other industrialized countries, has been recognized as a challenge to the primary care physician, the infectious disease specialist and public health authorities alike.

The clinical microbiology or immunology laboratory and the reference laboratory have an essential role to play in ensuring accurate documentation of group A streptococcal infections and are therefore vital to national programmes for the control of rheumatic fever, rheumatic heart disease, acute post-streptococcal glomerulonephritis and severe invasive group A streptococcal infections.

This manual provides a comprehensive, "user friendly" and well referenced guide to the procedures commonly required for laboratory evaluation of group A streptococcal infections. It includes advice not only on bacteriological methods, but also on serological evaluations and streptococcal antibody tests. Descriptions of other important techniques, such as the preparation of antisera for streptococcal serotyping (many of which cannot be purchased commercially), are especially valuable additions. The methods described are a compilation of the extensive experience in the two reference laboratories where the authors work, but procedures and suggestions from the published literature (with appropriate references) are also included. Although other methods have been used, those included in this manual are the ones that have been reported to be effective.

Streptococcal reference strains and sera that are not commercially available can be obtained either from the WHO Serum Bank, Minneapolis, MN, USA, or from the WHO Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. (The WHO Serum Bank is the result of a collaborative project by WHO Collaborating Centres in Prague, Minneapolis, New Delhi, Lyon and Rome, and the National Streptococcal Centre in St Petersburg.)

The World Health Organization has long been actively involved in promoting standardized techniques for the laboratory diagnosis of streptococcal infec-

tions. An initial set of guidelines (*Manual of microbiological diagnostic methods for streptococcal infections and their sequelae*, unpublished document WHO/BAC/80/1), prepared by Dr R.R. Facklam and the late Dr J. Rotta, was issued by WHO in 1980 and used extensively in laboratories around the globe. Following numerous advances in laboratory technology and in the understanding of group A streptococcal infections, the WHO Collaborating Centres for Reference and Research on Streptococci in Prague and Minneapolis have updated this document and extended its scope to include additional procedures useful to clinical, reference and research laboratories. WHO is grateful to the authors for their dedication in preparing this essentially new publication, thereby sharing their knowledge and expertise with laboratory and research workers throughout the world.

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Authors' introduction

In the identification and control of group A streptococcal infections, the role of the laboratory is crucial. This is as true of the microbiological laboratory, with responsibility for cultivation of group A streptococci from the upper respiratory tract, as it is of the clinical laboratory determining antibody titres against the organisms. Despite the development of new antimicrobial agents, experience over the past decade has shown that group A streptococcal infections are still important — and likely to remain so in the foreseeable future. At the close of the twentieth century understanding of these infections is incomplete, but their continuing public health significance serves to focus attention on the activities of both clinical and research laboratories. The WHO Collaborating Centres in Prague and Minneapolis have both noted growing numbers of requests for advice, for training of laboratory personnel, and for laboratory reagents (many of which are not commercially available).

This manual has been prompted largely by the resurgence of the clinical and epidemiological problems posed by group A streptococcal infections and by the volume of technical enquiries. We are confident that the experience gained in our two laboratories will be of value to others and contribute to the precision and standardization of diagnostic work elsewhere. The methods documented are those most frequently used in clinical microbiology and immunology and in research laboratories, but comprehensive guidance is provided for those working in more peripheral settings. Most methods are a compilation of the techniques used in our two laboratories and found to be effective, but there are a number of references to, and suggestions drawn from, the published literature. On occasion, two separate methods are described, one from Minneapolis and one from Prague; these reflect merely our different practices, and there is no intent to imply that one method is to be preferred to the other.

This manual represents an expansion of a somewhat more condensed WHO internal document (WHO/BAC/80/1) prepared in 1980 by Dr Richard Facklam and the late Dr Jiri Rotta. Although many of the classical techniques remain the same, much has changed in both clinical and research laboratories. This manual therefore contains a number of techniques not included in the 1980 document, as well as an extensive list of references that provide additional information for the interested user. Every effort has been made to present the content in a way that is understandable and accessible to readers at all levels.

We acknowledge with gratitude the guidance provided by the 1980 document prepared by Drs Facklam and Rotta; many of their original passages are quoted verbatim. Each of our manual's co-authors has contributed extensively to its content, but there are other colleagues, too numerous to mention individually, whose suggestions and generous provision of material we greatly appreciate.

We are especially grateful to Dr Evgueni Tikhomirov of WHO's Division of Emerging and Other Communicable Diseases Surveillance and Control, who has provided the encouragement and the opportunity for us to complete this

project, and to Mrs Sarah Ballance of WHO's Office of Publications for her careful editorial work on the manuscript. Thank you both.

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1. General introduction

1.1 The impact of group A streptococcal infections

Streptococcus pyogenes of Lancefield group A (group A streptococci) are common and important human pathogens all over the world. They are responsible for a wide variety of infections and their sequelae, usually, but not exclusively, occurring in children, adolescents and young adults. The infections may occur either endemically or as epidemics. The main portal of entry for group A streptococci and their principal site of residence in humans is the upper respiratory tract. Streptococcal pharyngotonsillitis is the most common of all bacterial throat infections. If accompanied by the typical rash, it is known as "scarlet fever." In most instances "strep throat" is a self-limiting infection, but it may progress to cause abscesses (in the tonsils or peritonsillar or retropharyngeal tissues) or cervical lymphadenitis, sinusitis, otitis media, mastoiditis, and even meningitis. In the lower respiratory tract, group A streptococci may cause pneumonia. A significant percentage of true pharyngeal infections, confirmed by a significant rise in streptococcal antibody titres, are clinically mild or even inapparent. They are, nevertheless, also associated with the risk of late sequelae, and may be active sources of spread of virulent streptococci, in contrast to "chronic carriers" (*1*).

Of the primary skin infections caused by group A streptococci, impetigo (pyoderma) is the most frequent, especially in tropical climates. Other manifestations include erysipelas, localized purulent infections from minor injuries and wounds, and cellulitis (including perianal cellulitis). Some of the rare but severe cases of necrotizing fasciitis or myositis have been linked with skin infections. The third site of primary streptococcal infections is the female genital tract: vulvovaginitis and puerperal fever, although uncommon, are still encountered in many countries.

Throat, skin and genital infections may develop into life-threatening septicaemia, streptococcal toxic shock syndrome, or metastatic suppurative infections such as arthritis, osteomyelitis, peritonitis or even acute endocarditis.

Group A streptococci are unique in initiating late non-suppurative sequelae, such as glomerulonephritis, which may occur after infection of either the throat or skin by nephritogenic strains, and rheumatic fever which follows throat infections only. Rheumatic heart disease is a major public health problem in many developing countries (*2*). Although it has become relatively rare among economically developed populations, sporadic, localized outbreaks of rheumatic fever do occur and serve as a reminder of this continuing threat (*3*).

Group A streptococci are the major cause of acute bacterial pharyngitis (*4*). Confident differentiation of streptococcal from non-streptococcal pharyngitis on the basis of clinical signs and symptoms alone is difficult, if not impossible. Reliable bacteriological methods of identification are therefore necessary for accurate diagnosis and appropriate therapy. Adequate antibiotic therapy essentially eliminates the risk of rheumatic fever, probably reduces the incidence of suppurative complications, speeds up recovery (if started early) and prevents further spread or extension of the infection.

It should be remembered that accurate diagnosis of acute rheumatic fever or post-streptococcal acute glomerulonephritis requires evidence of an antecedent infection with group A streptococci. This is best provided by using serological methods to demonstrate a rise in anti-streptococcal antibodies, although antibody determination may not be clinically useful at the onset of the illness or at the time of the initial attempt at diagnosis.

1.2 Structure and antigenic composition of group A streptococci

The cell structure of group A streptococci includes several important components. The outermost layer is the capsule consisting of hyaluronic acid. Streptococci differ greatly both in the amount of capsule produced and in their production of hyaluronidase, which destroys capsule. The cell wall surface, which is the outermost layer in the absence of the capsule, is covered with hair-like protrusions, or fimbriae, made up of the M, T and R protein antigens and lipoteichoic acid. The biological function of the R and T proteins is unknown, although they are very useful epidemiological markers. It has been shown that lipoteichoic acid facilitates the adherence of group A streptococci to mucous membranes. The M protein is a principal virulence factor of group A streptococci, providing protection from phagocytosis. Under the Lancefield classification scheme, nearly 80 antigenic M-protein serotypes have been officially recognized. In addition, a number of provisional M types have been described, and many strains carry M proteins that have not yet been characterized. Antibodies to M protein are associated with type-specific immunity. A model of the streptococcal M protein (5) is shown in Fig. 1. The C terminus of the coiled coil M protein acts as a membrane anchor. From the membrane anchor region towards the NH_2 terminus, the M protein is composed of a proline/glycine-rich region embedded in the cell wall, followed by four regions, or blocks, of repeating amino acids (arbitrarily designated A–D). The amino acid sequences of blocks B–D appear to be highly conserved among different M proteins. However, the A repeat block and a short sequence of amino acids at the N terminus are hypervariable and are thought to be unique for each M molecule (*6*, *7*). Opacity factor (OF) is a type-specific enzyme antigenically associated with certain serotypes of M protein. It produces opacity in the sera of different mammalian species and can be used to identify certain M types. At present, at least 27 of the officially recognized M types are known to produce opacity factor, but there are undoubtedly many more that have not yet been characterized.

The cell wall proper (the skeleton of the cell) consists of peptidoglycan (repeated units of *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by a short peptide) and C polysaccharide (a polymer of *N*-acetylglucosamine and rhamnose). Serological grouping is determined by the antigenic structure of this C polysaccharide. The antigens of the cytoplasmic membrane (beneath the cell wall) and the cytoplasm proper are not utilized in the present routine classification of group A streptococci.

Group A streptococci produce a number of toxins and enzymes. Among these are the antigenic streptococcal pyrogenic exotoxins (erythrogenic toxins A, B and C) which are responsible, among other things, for the scarlet fever rash. Streptolysin S — an oxygen-stable, non-antigenic toxin — is important because it produces haemolysis around streptococcal colonies growing on the surface of aerobically incubated blood agar plates. Streptolysin O is a reversibly oxygen-labile cytolysin with cardiotoxic potential. Since this haemolysin is not active in the presence of oxygen, it is expressed on aerobically incubated plates only by colonies growing in subsurface agar stabs. It elicits an anti-

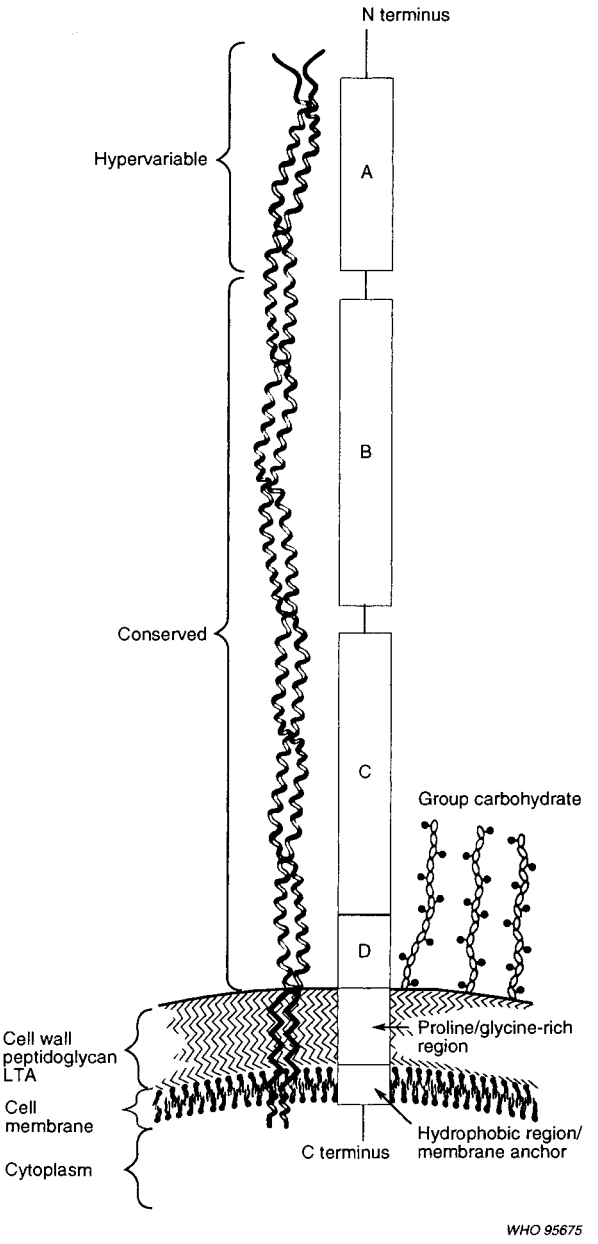

Fig. 1. Proposed model of the streptococcal M protein (*5*)
(Adapted by Stevens et al. (*5*) from: *Streptococcal M protein.* Vincent A. Fischetti. Copyright © 1991 by Scientific American, Inc. All rights reserved.)

body response (anti-streptolysin O) that is used diagnostically as evidence of infection. Antibodies to other extracellular antigens, such as deoxyribonuclease B (DNase B), hyaluronidase, streptokinase and nicotinamide adenine dinucleotidase (NADase), and antibody to the cell wall carbohydrate, antipolysaccharide, are also used in diagnostic laboratory tests. The most commonly used antibody tests for verification of group A streptococcal infection are the anti-streptolysin O and anti-DNase B tests. Both tests are commercially available, as is the anti-hyaluronidase test.

2. Specimen collection and transportation

2.1 Specimen collection

Swabs made of cotton wool or synthetic fibre are used for collecting specimens from the throat, nose, skin lesions, wounds or other affected body sites. If the site or lesion is not itself moist, the swab should be moistened with sterile water or saline before use.

For sampling the throat, the swab should be rubbed quickly but thoroughly over both tonsils (or tonsillar fossae) and over the posterior wall of the pharynx using light pressure. Heavy contamination of the swab with saliva should be avoided, since competitive inhibition of growth of group A streptococci by oral microorganisms present in saliva has been observed. Swabbing is best accomplished under direct vision and with the aid of a tongue depressor. If an exudate is present, it should be sampled.

For sampling the nose, the swab should be inserted into each of the anterior nares and rubbed around their interior. Taking both throat and nose swabs is advantageous whenever detection of small numbers of group A streptococci in the upper respiratory tract is important. In specific epidemiological circumstances (e.g. in hospital epidemics), group A streptococci may be shed from rectal, perianal or vaginal carriers (*8*, *9*); it may be necessary to sample these sites when this is suspected.

Serous material can be collected from skin lesions and wounds. Vesicles on the skin can be sampled after cleansing the surface with 70% alcohol and puncturing aseptically. In the case of impetiginous crusts, the lesion can be wiped with 70% alcohol, the crust removed aseptically with a sterile needle and the base of the lesion sampled with a moistened swab. Another method reported to be successful involves leaving the crust *in situ* and using the fingers to stretch the skin at both edges of the crust in order to express a droplet of the purulent material from underneath. This droplet should be collected on the tip of a dry swab, which should not touch the skin itself. Cleansing the lesion before this procedure is not thought to be necessary. Whatever method is used, it is always preferable to obtain material from fresh lesions, which tend to yield pure cultures of streptococci. Older lesions frequently yield both streptococci and staphylococci.

For erysipelas with an intact surface, needle aspiration from the leading edge of the lesion may be attempted by injecting a small amount (e.g. 0.1 ml) of sterile saline intradermally into the area and immediately drawing the fluid back into the syringe. However, the success rate in recovering group A streptococci is rather low using this technique. Because of the small amount of fluid recovered, it is best to express the contents of the needle directly onto a blood agar culture plate or into an enriched liquid medium, such as serum broth or blood broth. For bullous erysipelas, the blister fluid should be aspirated into a syringe after cleansing.

Aspiration into a syringe is the standard method of sampling in cases of peritonsillar abscess, purulent lymphadenitis, sinusitis, otitis media, osteomyelitis or other localized group A streptococcal infection.

2.2 Transport of specimens to the laboratory

Any delay between collection of a specimen and its examination in the laboratory increases the likelihood of false-negative results: immediate inoculation onto blood agar plates is best. If the delay is greater than a few hours (the time limit depends on the quality of the material used for swabbing, on environmental factors and on the nutrient media), a transport medium (e.g. Stuart's medium), the filter-paper strip method or the silica-gel transport system should be used.

2.2.1 Transport medium

For specimens that cannot be immediately plated, use of a transport medium is recommended to prevent loss of viability of the streptococci. If plating will be delayed for more than 48 hours, however, it is advisable to use either the filter-paper strip method or the silica-gel method described in sections 2.2.2 and 2.2.3 respectively. Culture transport systems using Stuart's transport medium are commercially available and have been successfully used for a number of years. Stuart's medium can also be prepared in the laboratory as described below (*10*).

Equipment and supplies

— Screw-capped test-tubes
— pH meter or indicator paper
— Autoclave

Reagents

— Anaerobic salt solution:	
chlorine-free (deionized) distilled water	900 ml
calcium chloride, 1% aqueous	20 ml
sodium glycerophosphate, 20% aqueous	100 ml
sodium hydroxide, 1 mol/litre (to bring the pH to 7.2)	12–15 ml
thioglycolic acid	2 ml
— Agar solution:	
chlorine-free (deionized) distilled water	1000 ml
agar	6 g
— Methylene blue, 0.1% aqueous solution	4 ml

Method

1. Mix the anaerobic salt solution ingredients, adding sufficient sodium hydroxide to bring the pH to approximately 7.2.
2. Melt the agar and add it to the anaerobic salt solution.
3. Check the pH and adjust to 7.3–7.4 if necessary.
4. Add 4 ml of methylene blue solution and mix well.
5. Dispense into screw-capped test-tubes (tubes should be nearly full) and autoclave at 121 °C for 15 minutes. After autoclaving and cooling, the medium should be colourless.
6. To use, the throat swab should be inserted into the medium, the shaft broken or cut off, and the tube tightly resealed for dispatch to the laboratory.

2.2.2 Filter-paper strip method

Drying of the sample on a filter-paper strip and maintaining it in a dry state can result in prolonged viability of group A streptococci (*11–13*). It also essentially maintains the relative prevalence rates of these bacteria and the remaining flora in the sample. During subsequent incubation of the strip on the surface of a blood agar plate, the paper is fully saturated with nutrient liquid from the agar. The organisms grow between the strip and the agar surface in a capillary layer of the broth. Under these conditions even otherwise weakly haemolytic group A streptococcal strains will usually produce distinct haemolysis.

Preparation of filter-paper strip kit

Equipment and supplies

— Filter paper (e.g. Whatman No. 1) cut into strips measuring 2 × 6 cm
— Glassine paper (non-absorbent paper resistant to steam sterilization) cut into strips measuring 3 × 13 cm
— Aluminium foil, 0.015 mm thick, measuring 10 × 20 cm and folded in half to form a double layer

Method

1. Mark the filter-paper strip on one side with a pencil.
2. Fold the glassine paper nearly in half but leave the upper lip somewhat longer to facilitate opening with the fingers without contaminating the filter-paper strip.
3. Insert the filter paper into the folded glassine paper so that the pencil mark is against the shorter side.
4. Wrap the glassine paper containing the filter paper in aluminium foil, so that the closure of the aluminium foil is on the plain side of the filter paper.
5. Autoclave the foil-wrapped packets at 121 °C for 30 minutes.
6. After autoclaving, allow the filter-paper kits to dry completely before using.
7. If the sterilized kits are kept completely dry, they may be held ready for use for many years without need for resterilization.

Transfer of sample onto the filter paper

Equipment and supplies

— Filter-paper kit previously prepared and sterilized
— Ball-point or felt-tipped pen for marking
— Cotton wool or synthetic fibre throat swab

Method

1. Label the kit with a ball-point pen (or a pencil) on the outer aluminium wrapping.
2. Unfold the aluminium foil wrapping. Check that the plain side (not marked with pencil) of the filter-paper strip faces upwards.
3. Collect the specimen with the swab.
4. Immediately transfer the specimen onto the filter-paper strip as shown in Fig. 2. Uncover the filter paper by lifting the upper lip of the glassine paper and apply the swab to the unmarked side of the filter paper, rolling it

Fig. 2. Transfer of streptococcal specimen onto filter paper for transport

under light pressure over as much of the filter-paper strip as possible. Touch the remaining parts of the strip repeatedly with the swab tip.

5. Let the upper lip of the glassine paper return to its original position, and allow the specimen to dry completely for 5–15 minutes, depending on the relative humidity of the environment.
6. Refold the outer aluminium foil wrapping. Keep the kit in a dry place at room temperature until delivery to the laboratory.

Comments

- The kit can be mailed in an ordinary envelope if permitted by local postal regulations.
- Culture of the dried specimen can be postponed for up to 7 days without significant decrease in streptococcal counts, even from sparsely colonized chronic carriers.
- Since humidity significantly decreases the viability of group A streptococci on filter-paper strips, this technique may fail in conditions encountered during rainy seasons in tropical and subtropical countries. This problem can be alleviated by keeping and sending the samples in a closed container with a desiccant (silica gel) to remove moisture.

Culture from the filter-paper strip

Equipment and supplies

— Forceps
— Bunsen or alcohol burner

— Blood agar plates (see section 3.1.3)
— Incubator (37 °C)

Method

1. Unfold the kit in the laboratory.
2. Remove the filter-paper strip with sterile forceps (flamed in the burner).
3. Lay the filter-paper strip *face down* (pencil-marked side up) on the surface of a blood agar plate. The paper should be close to the edge of the plate, and no air bubbles should remain beneath the filter-paper strip.
4. Incubate the plate aerobically for 4–5 hours at 37 °C.
5. After this initial incubation, remove the strip with sterile (flamed) forceps and lay it down for a few seconds on the middle third of the same plate. Finally, transfer the strip onto the remaining third of the same plate, always face down.
6. Incubate at 37 °C overnight.
7. After overnight incubation, examine for β-haemolytic streptococci.

Comments

- The first and the second areas of contact of the filter-paper strip yield typical separated colonies of bacteria. The final overnight area of contact usually yields confluent microbial growth. If β-haemolytic streptococci are present under the filter paper, they will be manifest only as clear, sharp zones of haemolysis. The mere appearance of this characteristic haemolysis signifies the presence of streptococci. Only exceptional strains of *Staphylococcus aureus* mimic this streptococcal β-haemolysis beneath the filter-paper strip.
- If no streptococcal colonies are found in the first two areas of the plate but haemolysis is evident under the filter paper, prepare a subculture from at least one spot of β-haemolysis. Remove the filter-paper strip from its position only shortly before the isolation trial. It is sufficient to touch a wire loop on the centre of a typical haemolytic zone and transfer to a fresh blood agar plate.
- If no haemolytic zone is seen anywhere on the original plate, prolong the incubation for a further 24 hours, replacing the strip in its "overnight" position.
- Results obtained by the filter-paper strip technique have been found to be equivalent to the combined results from immediate plating of the swabs and their additional incubation in enrichment media. A semi-quantitative reading of the culture results is possible in the same way as in the classical primary plating of specimens.

2.2.3 Silica gel desiccated swabs

The silica-gel system is based on the filter-paper strip method but eliminates the need for drying specimens before packaging and shipping (*14*). As discussed in section 2.2.2, the presence of moisture will have a deleterious effect on the viability of the streptococci during the transport of specimens on filter-paper strips. This problem can be eliminated by placing the specimens in silica-gel desiccant. Although there no longer seems to be a commercial source of prepared silica-gel mailing containers, they can be easily fabricated in the laboratory as described below.

Equipment and supplies

— Screw-capped tubes long enough to hold throat swabs
— Silica gel, mesh size 12–28, grade 08
— Cotton wool or synthetic fibre throat swabs
— Autoclave
— Dry-heat oven
— Incubator

Method

1. Add sufficient silica gel to the tube to cover the head of a throat swab (depth of approximately 2 cm).
2. Loosely cap the tube and autoclave at 121 °C for 15 minutes to sterilize.
3. Dry the tube (with the cap loosened) in a dry-heat oven.
4. When cool, securely tighten the cap and store until needed.
5. After taking a throat specimen, immediately insert the swab into the tube so that the head is completely covered by the silica gel; securely tighten the cap before dispatch to the laboratory.
6. On receipt in the laboratory, remove the swab carefully from the tube. Dip the swab into a tube of Todd–Hewitt broth and then streak onto a blood agar plate. Return the swab to the Todd–Hewitt tube for overnight incubation (35–37 °C) and follow-up culturing.

Comments

- The silica gel can be blended with indicator blue (approximately 3–5%, grade 46) as a dryness indicator.
- Potentially contaminated particles of silica gel may cling to the swab and pose a possible biohazard risk in the laboratory. The swab should be removed from the tube in such a way that any loose particles are contained within the tube.

2.2.4 Transport of group A streptococci on agar

Although transport of streptococci on blood agar plates is common, the method is not ideal. Unless the plate is tightly sealed, there is a risk of the agar drying, with attendant loss of viability of the streptococci. Also, the rough handling that mailed parcels frequently receive may result in damage to the plates. In addition, dispatch of cultures on blood agar plates is illegal in many countries. It is therefore better to use either blood agar or chocolate agar slants or agar "deeps" prepared in screw-capped tubes or small vials. These can be well sealed to prevent dehydration and, if protected with adequate padding, are quite resistant to damage from rough handling. These methods are best suited for the transport of pure cultures of streptococci, and have been very successfully used in many studies.

Blood and chocolate agar slants are readily available from commercial sources or can be easily prepared in the laboratory. Slants have the advantage that growth of the strain can be readily seen, and it is often possible to detect contamination on the slant before dispatch. A disadvantage is that the exposed agar surface is also subject to the deleterious effects of dehydration. Under normal conditions, however, with well sealed tubes and relatively short transport times, dehydration is not a significant problem. Agar deeps can be prepared by nearly filling a small vial with blood agar or blood agar

base without blood. The advantage of not using blood is that it is easier to visualize the growth of streptococci in the agar. The deep is inoculated by removing several colonies from a blood agar plate with an inoculating loop and then stabbing the loop vertically down the full depth of the agar. When the loop is removed the agar closes around the stab, forming an environment that is excellent for growth and very stable in moisture content. Under these conditions viability of streptococci will frequently remain good for several weeks.

3. Culture, recognition and storage

3.1 Culture media

Streptococci are fastidious in their nutrient requirements. In addition to a complex nutrient source, such as meat infusion, and a glucose source, culture media should contain buffers to prevent acidification and subsequent killing of the streptococci during growth.

3.1.1 Non-selective broth media

Todd–Hewitt broth

The standard medium used for the culture of group A streptococci is modified Todd–Hewitt broth (*15*), which is composed of beef heart infusion, Neopeptone, glucose, salt and buffers, and is commercially available. Todd–Hewitt broth can also, if necessary, be produced from raw ingredients (*16*). When streptococci are grown for M typing or for vaccines for production of anti-M antibodies, Todd–Hewitt broth with Neopeptone is necessary to inhibit production of active proteinases that digest and destroy M protein. Neopeptone is also reported to stimulate the synthesis of M protein (*17–19*). It has been reported that addition of 2% yeast extract to Todd–Hewitt broth improves the yield of streptococci by nearly three times, and that 3% yeast extract plus shaking the culture increases wet weight yield by 4–5 times with a concomitant increase in the yield of M protein (*20*).

Equipment and supplies

— Heater
— Autoclave
— Funnel
— Filter paper or cotton wool

Reagents

— Beef or beef heart, fat-free
— Distilled water
— Neopeptone
— Sodium hydroxide, 10 mol/l
— Glucose
— Sodium bicarbonate
— Sodium chloride
— Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)

Method

1. Mince the beef or beef heart and to every 450 g add 1 litre of distilled water. Stir well and keep at 4 °C overnight.
2. Heat to 85 °C and maintain at this temperature for 30 minutes with occasional stirring.
3. While still hot, filter through filter paper or cotton wool. Any loss through evaporation can be made up with distilled water.

4. Add 20 g of Neopeptone to each litre and adjust to pH 7.0 with NaOH, 10 mol/l (about 3 ml per litre will be needed).
5. To each litre of medium add:

sodium bicarbonate	2 g
sodium chloride	2 g
glucose	2 g
disodium hydrogen phosphate	1 g

6. Boil the medium for 15 minutes.
7. While still hot, filter through filter paper.
8. To sterilize, autoclave at 115 °C for 10 minutes.
9. Bring the final pH to 7.8, and store the medium at 4 °C.

Serum broth

Serum broth is prepared by adding 20% (v/v) normal calf or horse serum to Todd–Hewitt broth and distributing it aseptically into test-tubes of the desired volume (e.g. 6 ml). Absence of contamination is checked by incubation overnight at 35–37 °C and once more overnight at room temperature. Tubes without any sign of contamination are then kept at 4 °C until required for use, and may be stored this way for many weeks if protected from evaporation (e.g. by placing in a plastic bag).

Blood broth

Blood broth is prepared from Todd–Hewitt broth by adding 3–5% (v/v) defibrinated sheep, horse or rabbit blood. It should be checked for sterility and stored as described for serum broth.

3.1.2 Selective broth media

Pike's medium

Pike's medium (*21*) has been found by some investigators to increase the yield of group A streptococci from throat cultures by inhibiting the growth of staphylococci and Gram-negative bacteria.

Equipment and supplies

— Culture tubes
— Pipettes, 2.0 ml, 0.15 ml and 0.10 ml
— Cotton wool or synthetic fibre throat swabs
— Incubator

Reagents

— Blood infusion broth. Pike used beef heart infusion broth containing 1% tryptose, 0.02% glucose and 5% rabbit blood
— Sodium azide, concentration 1:1000 (0.1 g/100 ml) in an aqueous solution, autoclaved at 121 °C for 15 minutes
— Crystal violet, concentration 1:25000 (4 mg/100 ml) in an aqueous solution, autoclaved at 121 °C for 15 minutes

Method

1. Prepare tubes with 2-ml aliquots of blood broth.
2. Add 0.15 ml of the autoclaved solution of sodium azide to each tube (final concentration 67 µg/ml).

3. Add 0.10 ml of the autoclaved solution of crystal violet to each tube (final concentration 1.7 μg/ml).
4. Collect specimen with a swab.
5. Place the swab in a tube containing Pike's medium and incubate overnight at 35–37 °C.
6. Subculture the broth to a standard blood agar plate and incubate as for a primary culture.

Comment

- Pike observed that the broth began to lose its growth-inhibiting ability 2 days after the addition of the sodium azide.
- Nakamizo & Sato reported good results using an enrichment broth containing sodium azide but without crystal violet. Additional components of their broth were yeast extract, large amounts of NaCl, activated charcoal, saponin, urease and camphor (22).

3.1.3 Non-selective agar media

Standard agar media

Many agar media give satisfactory growth of group A streptococci, especially when supplemented with blood or serum. Tryptic soy agar with 5% sheep blood is commonly used, and many variations have been developed and marketed by commercial suppliers of laboratory products. Agar media can also be produced by purchasing and combining individual components (agar, meat extract, buffer, etc.). However, whether media are produced in the laboratory or purchased from a commercial manufacturer, each new batch should be tested for its ability to support good growth of streptococci with typical colony morphology and haemolytic properties. Blood agar media used in the evaluation of haemolysis should not contain any added glucose, which will inhibit β-haemolysis, and should have a gelling temperature ≤48 °C.

Equipment and supplies

— Petri dishes
— Pipette or syringe for adding blood to agar
— Autoclave
— Water-baths, 37 °C (optional) and 48–50 °C

Reagents

— Blood agar base
— Defibrinated blood (sheep, horse, rabbit — see section 3.4). Collect blood in a sterile flask containing 1–2 layers of glass beads (diameter 5 mm). Stir slowly until clotting is complete (≈10 minutes). Decant the defibrinated blood into a sterile container, and store at 4 °C for up to 5 days. Check sterility before use.

Method

1. Prepare blood agar base according to manufacturer's instructions, autoclave at 121 °C for 15–30 minutes and cool in a water-bath to 48–50 °C. Some laboratories prefer to dissolve the agar base by heating to 100 °C before autoclaving.

2. Warm blood to room temperature (20–22 °C) or, if preferred, to 37 °C.
3. To the sterile blood agar base at 48–50 °C add 5–7% warmed blood.
4. Mix thoroughly and pour 3–4 mm thick. Avoid bubbles in the agar surface.
5. Allow plates to harden thoroughly on a level surface before moving them. One plate from each batch should be incubated at 35–37 °C as a sterility control.
6. Plates can be left on the bench overnight to dry. Store sealed in a plastic bag at 4 °C. Usable life of stored plates will depend on the quality of the blood, the composition of the agar base and the storage conditions.

3.1.4 Selective agar media

Selective media can also be obtained commercially or prepared as described below.

Crystal violet agar

Based on the same principle as Pike's medium described above (section 3.1.2), blood agar plates containing crystal violet can be very useful in situations where cultures are likely to be overgrown with staphylococci, for example, in cultures of skin and skin lesions, and in pharyngeal and nasal cultures in populations that commonly yield high numbers of staphylococci from these sites. High concentrations of haemolytic staphylococci may completely mask streptococcal haemolysis.

Equipment and supplies

— As described for standard agar media
— Pipette (5 ml) for adding crystal violet to agar

Reagents

— As described for standard agar media
— Aqueous solution of crystal violet, concentration 1:10 000, autoclaved (at 121 °C for 15 minutes)

Method

1. Prepare blood agar base as for normal blood agar plates, autoclave at 121 °C for 30 minutes and cool to 48–50 °C.
2. Warm blood and add to blood agar base as for normal blood agar plates.
3. To 500-ml aliquots of blood agar, add 5 ml of the sterile crystal violet solution (final concentration 1:1 000 000 (1 µg/ml)).
4. Mix thoroughly and pour as for normal blood agar plates.

Columbia agar with colistin and either nalidixic or oxolinic acid

Streptococcal cultures from sources likely to carry Gram-negative bacteria are often more successfully cultured using inhibitory antibiotics. Columbia 5% blood agar containing colistin (10 µg/ml) and nalidixic acid (15 µg/ml) has given good inhibition of Gram-negative organisms (*23*), but it does not inhibit either staphylococci or coryneform bacteria. However, Columbia 5% blood agar containing colistin (10 µg/ml) and oxolinic acid (5 µg/ml) will inhibit staphylococci and coryneform as well as Gram-negative organisms (*24*).

Sulfamethoxazole–trimethoprim agar

Some investigators have found that use of a combination of sulfamethoxazole and trimethoprim in a standard primary blood agar plate may increase the recovery of group A streptococci (25, 26). This medium is composed of tryptic soy agar with 5% sheep blood supplemented with sulfamethoxazole (23.75 μg/ml) and trimethoprim (1.25 μg/ml).

3.2 Culture methods

For proper inoculation of swabs, it is preferable to use an entire blood agar plate. However, if inoculation is done carefully, one half of a standard size plate can be used. Roll the swab specimen across an area measuring approximately 3 × 2 cm near the edge of the plate and spread three or four sets of streaks from this with a sterilized wire loop to obtain well spaced colonies (Fig. 3). Additional inoculation by a few oblique stabs into the agar with the loop is important. This allows growth below the agar surface under nearly anaerobic conditions and results in the formation of more distinct zones of β-haemolysis by strains that are normally weakly haemolytic under aerobic incubation; subsurface haemolysis is due to oxygen-labile streptolysin O. The blood agar plates must not be too dry if successful recovery of small numbers of group A streptococci is to be achieved.

Primary plating of samples rich in group A streptococci is fully satisfactory for the diagnosis of acute streptococcal infection. For detection of the bacteria from sparsely colonized sources, like newly diagnosed cases of acute rheumatic fever or acute glomerulonephritis, the filter-paper strip technique is very useful. Alternatively, primary plating should be supplemented by the use of either selective or enrichment media (Pike's medium, serum broth or blood broth). After preliminary plating, incubate the swab in an enrichment medium overnight at 35–37 °C and plate it again using a fresh blood agar plate. This is done by transferring a very small droplet to the plate and spreading it thoroughly with a loop to obtain separated colonies. If the enrichment broth medium used is non-selective, final plating on a selective agar medium may be beneficial. For healthy carriers, the use of an enrichment medium improves the detection rate of group A streptococci by 15–20% compared with primary plating performed immediately after swabbing.

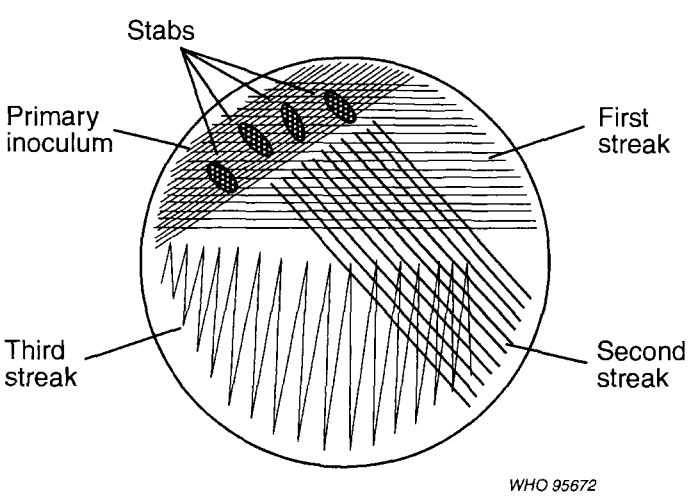

Fig. 3. Differential plating technique for primary cultures of β-haemolytic streptococci

Liquid samples supposedly not rich in group A streptococci, such as pus from abscesses or fluid aspirated from the spreading edge of erysipelas, can be inoculated directly into enriched liquid media. If the collected sample is small, also rinse out the syringe with 1 ml of the same medium. Incubate at 37 °C for 15–18 hours (longer if necessary) before plating onto blood agar.

Although saliva cultures may yield haemolytic streptococci, careful studies have shown that throat cultures may be more reliable (27). Cultures of undiluted saliva (0.1 ml spread evenly over a blood agar plate) frequently yield a heavy overgrowth of oral bacteria, so that typical colonies of group A streptococci are rarely formed from samples containing fewer than 10^6 chains per ml.

For investigations whose sole objective is recovery of β-haemolytic streptococci, selective culture media are useful, for example crystal violet blood agar, which inhibits the growth of staphylococci, for nose swabs and for samples from skin lesions. More complex selective media containing antibiotics will inhibit Gram-negative organisms as well as almost all non-streptococcal Gram-positive organisms without any adverse effect on the recovery of streptococcal species. By reducing or even eliminating the overgrowth of competing bacteria, selective culture media can enhance the likelihood of success from samples containing only small numbers of group A streptococci.

3.3 Incubation conditions

Overnight incubation of appropriately inoculated samples on blood agar plates (preferably using sheep blood) at 35–37 °C under aerobic conditions is the generally accepted standard for bacteriological examination of most specimens. The recovery rate may be somewhat increased by incubation of inoculated plates (of plain or selective blood agar) in an atmosphere containing 5–10% of carbon dioxide or under anaerobic conditions. Anaerobic incubation supports the growth of streptococci and has some inhibitory effect on staphylococci, *Moraxella* (*Branhamella*), *Haemophilus* and *Corynebacterium*. It also enhances the formation of haemolytic zones by allowing expression of the oxygen-labile streptolysin O. However, the difference between aerobic and anaerobic incubation is not significant for samples with abundant group A streptococci. The temperature of incubation should not exceed 37 °C, since higher temperatures may inhibit the growth of some group A strains.

It is good practice to prolong incubation for an additional 24 or even 48 hours for all plates that show no growth of β-haemolytic streptococci after the initial overnight incubation.

3.4 Recognition of haemolytic streptococci by culture

Determining the presence of group A streptococci by culture depends primarily on the identification of haemolytic colonies on the surface of blood agar plates. Colonies of different strains of haemolytic streptococci, as well as other haemolytic bacterial species, differ from one another in size, form and colour, and in the character of the haemolysis.

Typical group A streptococcal colonies are mostly circular in shape with an entire edge and range from 0.5 to 2 mm or more in diameter. They appear in three main forms: mucoid, matt and glossy. Mucoid colonies are usually large and glistening with the appearance of droplets of water (Fig. 4(*a*)). They are viscous when touched with a loop and often form confluent growth. They are

formed by strains producing large amounts of hyaluronic acid capsule. Matt colonies, also called post-mucoid colonies, are flat with an uneven rough surface and often develop as a result of drying (collapse) of mucoid colonies (Fig. 4(*c*)). Glossy colonies are smaller than the other forms. They are domed with a shiny surface and are formed by strains that do not produce capsule during growth (Fig. 4(*d*)). In addition to these named forms, several intermediates may be differentiated (Fig. 4(*b*)). Traditionally, strains growing as glossy colonies were thought to produce less type-specific M protein and were therefore considered less virulent than mucoid or post-mucoid colony forms. However, there are many exceptions to this relationship. The mucoid property of a strain is now known to be due to the presence of hyaluronic acid capsule and not directly related to the presence of the more important virulence factor, M protein.

The size of the haemolytic zone around surface colonies of group A streptococci is usually 2–4 times the diameter of the colony; in some strains the zone is much wider, while in others no more than a narrow ring is present. The haemolytic effects of β-haemolytic and α-haemolytic streptococci grown on a sheep blood agar plate are compared in Fig. 5. There is complete clearing of

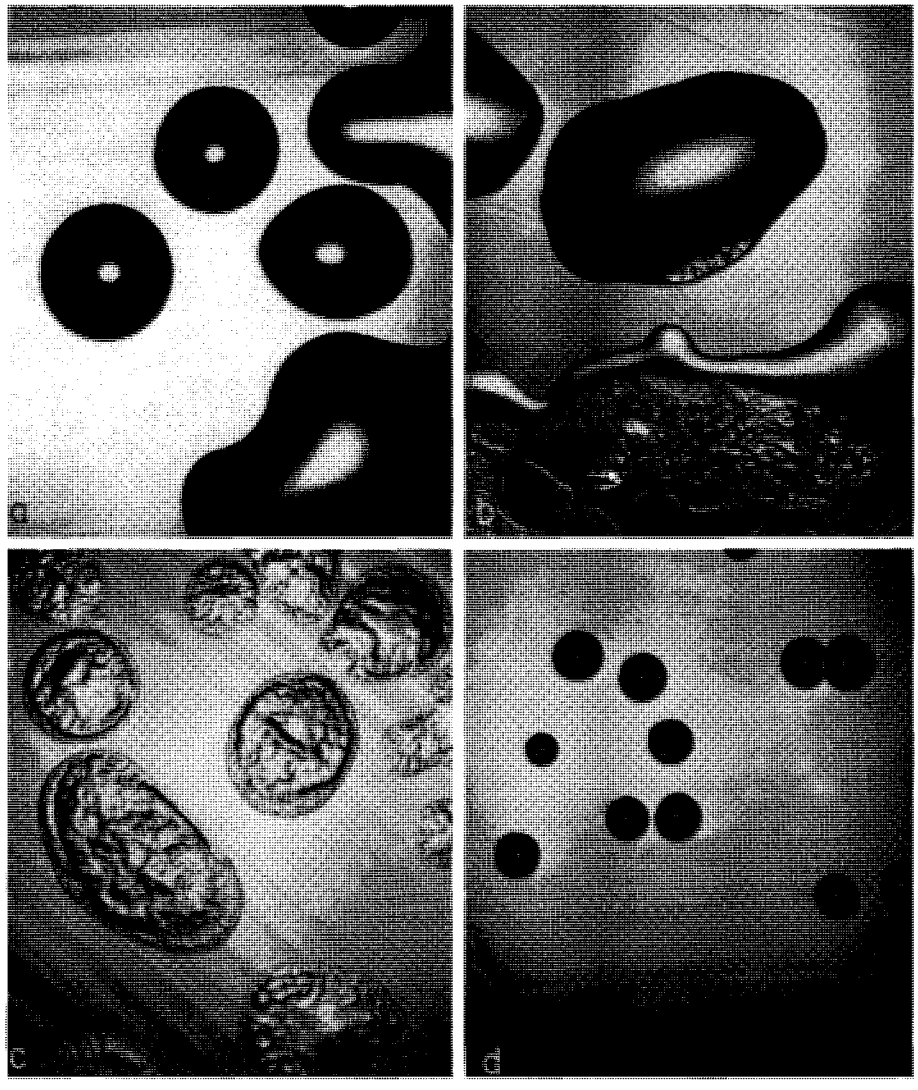

Fig. 4. Morphology of group A streptococcal colonies: (*a*) mucoid; (*b*) intermediate; (*c*) matt; (*d*) glossy (*28*)

(Photographs from Dr A.T. Wilson, Alfred I. duPont Institute of the Nemours Foundation, Wilmington, DE, USA. Used with permission.)

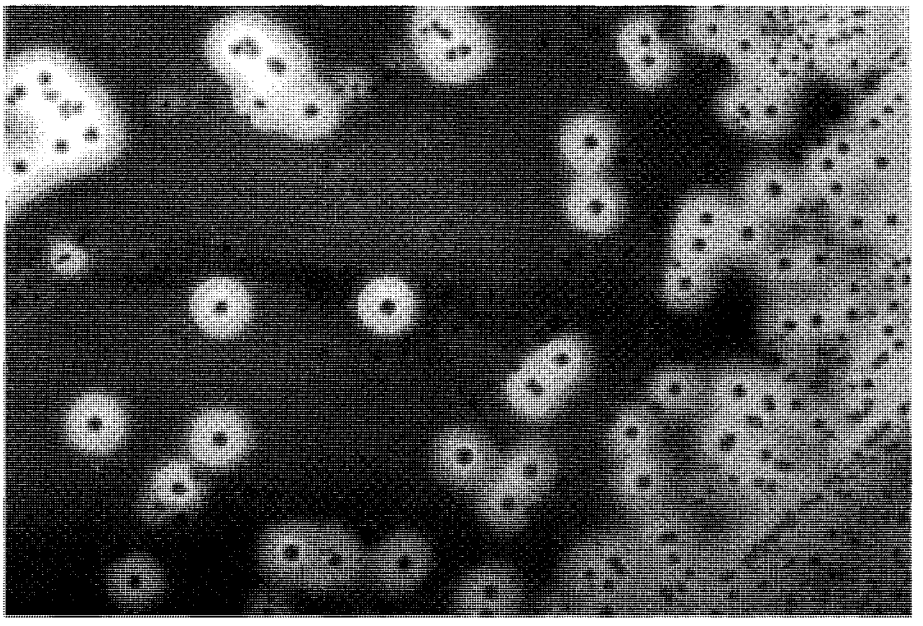

Fig. 5. Comparison of the haemolytic properties of β-haemolytic and α-haemolytic streptococci

the blood around the colonies of the larger β-haemolytic streptococci, while the blood surrounding the smaller colonies of α-haemolytic streptococci is discoloured but not completely lysed. Most strains of group A streptococci produce complete lysis of red blood cells. In some strains, however, a green discoloration resembling α-haemolysis is seen around the colonies, which develops into complete lysis only after prolonged incubation. This phenomenon has been reported to be due to inhibition of haemolysis by the opacity factor produced by some group A strains (*29*). Strains that produce no haemolysis under aerobic incubation have also been described (*30, 31*).

In assessing the morphological or haemolytic characteristics of streptococcal strains, it must always be remembered that both properties are strongly influenced by agar media composition, agar thickness, blood concentration, the animal species used as the blood source and the atmosphere of incubation. Sheep blood is preferred to horse or rabbit blood, since it does not support the growth of *Haemophilus haemolyticus*, a bacterium that might be confused with group A streptococci on blood agar culture. Human blood is generally not satisfactory for use in blood agar plates for identification of streptococci: blood obtained from blood banks may contain substances (antibiotics, citrate, etc.) that could inhibit either the growth or the haemolytic properties of streptococci. The presence of antibodies in human blood (anti-streptolysin O, anti-M protein, etc.) is also undesirable. Since important morphological characteristics may change from one batch of blood agar to another, colonies exhibiting different morphologies should be compared by inoculating each onto separate sections of the same plate, each spread sufficiently to obtain well separated colonies.

Colonies of group A streptococci are often indistinguishable from those of other groups of β-haemolytic streptococci, especially groups C and G, and serological methods are required for definitive identification. The presence of colonies of different morphological or haemolytic characteristics in the same blood agar culture may indicate a mixed culture of two or more unrelated strains of haemolytic streptococci belonging to different serological groups and/or different group A serotypes. The simultaneous presence of more than

one strain of β-haemolytic streptococci, although rarely detected in acute infections, can occur, especially in communities with a high prevalence of group A streptococci. Careful examination of colony morphology and haemolytic characteristics is therefore essential both for accurate laboratory diagnosis of streptococcal infection and for selection of strains for streptococcal research. It is vital that strains collected for research studies are pure, unmixed cultures.

If difficulties are encountered in differentiating streptococci from other haemolytic bacteria such as *Haemophilus, Corynebacterium, Staphylococcus* or *Moraxella* (*Branhamella*), a microscopic examination of Gram-stained smears from suspect colonies may be helpful. The catalase test is also often helpful in differentiating streptococci, which are always catalase-negative, from catalase-producing species.

3.5 Atypical morphological forms

Occasionally, colonies of haemolytic streptococci that do not match the descriptions given above are observed in primary cultures grown on blood agar plates. Three morphological and cultural variations that could cause confusion in the diagnosis of group A streptococci deserve special mention.

(1) Minute colonies of β-haemolytic streptococci may be encountered which, when grown and serologically grouped, are found to react with group A antiserum. Some, but not all, streptococci with this morphology require an increased concentration of carbon dioxide for growth. Even though these organisms carry cell-wall carbohydrate antigens apparently identical to those in typical large-colony forms of Lancefield group A strains, they are considered by most taxonomists to be a separate species. These minute streptococci carrying group A antigens have historically been classified by British taxonomists as *Streptococcus milleri* and by United States taxonomists as *S. anginosus*, group A (*32–36*). The current system of streptococcal nomenclature, in both Europe and the USA, divides the "milleri aggregate" into three species (*37*), two of which, *S. constellatus* and *S. anginosus*, may carry the Lancefield group A antigen (A. Efstratiou and R. George, personal communication). Most importantly, however, streptococci exhibiting this morphology but reacting with group A antiserum are not considered to be *S. pyogenes* and not thought to be associated with the serious infections (or sequelae) caused by group A streptococci.

(2) Group A streptococci have been reported to occur as nutritional variants and either to grow poorly or not to grow at all on the surface of aerobically incubated standard blood agar plates (*38–41*). In some cases, incubation in an atmosphere containing increased carbon dioxide will allow growth with typical morphology. In other instances, special supplementation of the media may be required. Occasionally these variants grow on blood agar only as satellites around other normal flora bacterial colonies. Some of these atypical forms have been shown to carry not only group A carbohydrate antigen, but also recognized T-protein and M-protein antigens, and are considered to be true group A streptococci (*39, 41*).

(3) Group A strains have been isolated that lack the ability to haemolyse blood when grown on blood agar plates (*30, 31, 42*). Of particular interest is the report from the Lowry Air Force Base in the USA, where a non-haemolytic, mucoid, group A strain belonging to type M18 was associated with an outbreak of rheumatic fever (*30*). Since identification of group A streptococci is usually based on recognition of haemolytic properties, the

existence of potentially "rheumatogenic" or nephritogenic non-haemolytic strain variants requires particular diligence on the part of the clinical microbiologist to ensure accurate diagnosis.

3.6 Reporting of culture results

The result of a primary plating may be reported as a semi-quantitative evaluation of the amount of β-haemolytic streptococcal growth. Several schemes for doing this are in common use. One scheme uses three categories and is defined as follows:

1+ <20 colony-forming units (CFU)
2+ >20 CFU, but streptococci not predominant
3+ streptococci predominant or pure culture

Another commonly used scheme uses four categories as follows:

1+ <10 CFU
2+ >10 but <50 CFU
3+ >50 CFU but streptococci not predominant
4+ streptococci predominant or pure culture

In contrast to the filter-paper strip technique, the culture results from swabs delivered in transport media (e.g. Stuart transport medium) or from growth in selective or enrichment media can be interpreted only in terms of the presence or absence of β-haemolytic streptococci.

True streptococcal infections of the pharynx (clinical as well as asymptomatic) often yield large numbers of β-haemolytic streptococcal colonies if adequate techniques are used. Throat swabs from healthy carriers may yield reduced numbers of colonies. Neither finding is entirely consistent, however, and the degree of positivity of the culture is not a reliable means of differentiating a true streptococcal infection from a chronic carrier (*43*). Heavy pharyngeal colonization by β-haemolytic streptococci may persist for several weeks. A newly presenting pharyngitis case with a positive culture of streptococci may in fact represent only a case of non-streptococcal pharyngitis in a "carrier" of streptococci. On the other hand, a negative culture result may be reported for a sample taken from a true streptococcal infection if appropriate techniques for obtaining and processing the specimen and interpreting the culture are not followed.

3.7 Preservation of streptococci

Storage of β-haemolytic streptococci on blood agar plates is one possible means of maintaining strains for subculture. Although many streptococcal chains remain viable in colonies for several weeks when kept at refrigerator temperature in a sealed Petri dish to avoid drying, such storage should ideally be limited to less than 2 weeks. Investigators storing strains in this way should be aware that subcultures derived from older colonies may be at increased risk of genetic change.

In order to preserve the genetic integrity of streptococcal strains, unnecessary laboratory passage or prolonged storage in conditions that allow continued metabolism should be avoided. For subculture, a liquid medium (e.g. serum broth) is preferred. Blood agar culture should, as a rule, be used only to check strain purity or to provide colonies to be picked for a new broth culture.

When a specific streptococcal strain is required in the laboratory for more than a few days, deep-frozen aliquots of a serum-broth culture, preferably stored at ≤–70 °C, are best. It is also possible to maintain strains frozen on filter-paper strips. Viability remains good for several months at –20 °C and for considerably longer at –70 °C. For long-term preservation of strains, freeze-drying — if properly done — usually gives the best results. Observance of these recommendations is usually conducive to long-term maintenance of desirable properties of strains and obviates the need for artificial enhancement by such techniques as mouse passage or passage in human blood.

3.7.1 Frozen log-phase aliquots of a serum-broth culture

Equipment and supplies

— Culture tubes containing 5 ml of serum broth (Todd–Hewitt broth with 20% normal calf or horse serum)
— Freshly grown blood agar plate culture of the strain to be preserved
— Sterile wire inoculating loop
— Vials suitable for freezing at low temperatures
— Incubator
— Freezer, at –20 °C or –70 °C

Method

1. Heavily inoculate 5 ml of serum broth in a culture tube with well separated colonies of the freshly grown culture.
2. Incubate for 4–6 hours at 37 °C (log phase).
3. Distribute the culture in aliquots of 0.2–0.5 ml.
4. Freeze the aliquots quickly and store at –70 °C.
5. To use, one aliquot is rapidly thawed in a water-bath at 30–37 °C and used as an inoculum.

Comments

- The presence of serum in the medium protects the bacterial cells during the freezing and thawing periods.
- Overnight serum-broth cultures of hundreds of strains (in 0.5-ml samples) have been preserved undamaged for more than 10 years by maintaining continuously at –70 °C. Log-phase cultures as described above should provide even better survival rates. A temperature of –20 °C, although less efficient, will generally maintain viability for between several months and several years.

3.7.2 Drying on filter-paper strips

Equipment and supplies

— Filter-paper strip package (see section 2.2.2)
— Freshly grown blood agar plate culture of the strain to be preserved
— Sterile wire inoculating loop

Method

1. Using the wire loop, transfer 10–15 colonies of streptococci from the freshly grown blood agar plate culture onto one half of the filter-paper strip, spreading colonies thoroughly by using slight pressure.

2. Repeat the process, spreading additional colonies onto the remaining half of the strip.
3. Let the filter paper dry for at least 5–10 minutes before refolding the outer aluminium foil wrapping (see section 2.2.2).
4. Store in a dry place.

Comment

- Streptococci dried on a filter-paper strip retain good viability for at least 1 month at room temperature if the strip is maintained in a dry environment (suitable for mailing between laboratories). Viability is maintained for at least several months if the strip is frozen at –20 °C, and for much longer periods (possibly years) if it is maintained at –70 °C.

3.7.3 Freeze-drying (lyophilization)

Optimal results have been obtained with serum-broth cultures in logarithmic growth phase (approximately 10^9 CFU/ml). Volumes of 0.15 ml of such a culture are freeze-dried and sealed under vacuum. Maintenance of a vacuum in the ampoule during storage is essential for long-term viability of the lyophilized sample. The freeze-dried strains can be stored at room temperature although lower temperatures will further prolong viability.

To revitalize the freeze-dried culture, the sealed ampoule may be conveniently opened as follows. Place a drop of cool water on the tip of an ampoule heated (over a Bunsen) to a high heat, to produce tiny cracks in the glass. This removes the vacuum inside the ampoule and the vial can be opened by a gentle knock on the cracked portion of glass. Dissolve the freeze-dried contents immediately with an excess of serum broth (0.5 ml) and inoculate onto a blood agar plate and/or into a larger volume of enrichment broth.

4. Determination of Lancefield serological group

4.1 Introduction

A clinical microbiology report that indicates only the presence of β-haemolytic streptococci is grossly inadequate. Accurate clinical diagnosis depends on knowledge of the specific serological group of the streptococci involved in the infection. Dr R.C. Lancefield first developed the method of classifying streptococci into such groups. Although group A to V (excluding I and J) are currently recognized, plus provisional groups W–Z (*44*), it is the group A strains that are responsible for the vast majority of serious human streptococcal infections. Some haemolytic streptococci that do not belong to group A are also capable of causing primary infections of the respiratory tract or skin, and sometimes even serious suppurative or systemic infections, but they are not associated with the risk of rheumatic fever or (with very rare exceptions) of acute glomerulonephritis. Hence, the therapeutic and prophylactic implications of group A and non-group A streptococcal infections are clearly different.

The group-specific antigen of most streptococci is a polysaccharide located in the cell wall (the group antigen of groups D, N and Q is a teichoic acid) and can be extracted by a number of methods. Its presence can then be demonstrated using group-specific antisera by methods such as precipitation, agglutination, immunofluorescence (*45*) or enzyme-linked immunosorbent assay (ELISA) (*46*).

A number of different extraction methods for group-specific polysaccharides have been described, including extraction with hydrochloric acid, formamide or nitrous acid, or by autoclaving a suspension of group A streptococci in saline (see section 4.3). Extraction with enzymes, including *Streptomyces albus* enzyme (*47*), pronase (*48*) or phage-associated lysin (*49*), has also been described. Although most group-antigen extraction methods require varying volumes of an overnight broth culture, the nitrous acid and enzyme extraction methods can be performed with much smaller volumes and have been successfully used with a loopful of colonies harvested from a blood agar plate.

Precipitation reactions with extracted polysaccharide may be performed as a ring test, a capillary test, a double-diffusion test or counterimmunoelectrophoresis (*50*) in agar gel or on cellulose acetate membrane. Agglutination reactions may be performed either as co-agglutination, using group-specific antibody bound to protein A containing staphylococci, or as latex agglutination.

Screening tests for presumptive group identification of streptococci have also been developed. The available tests for group A streptococci include the bacitracin disc and PYR tests (see section 4.2). It should be recognized that these are not totally reliable, so confirming tests should be performed.

Antisera for serological grouping are commercially available from a number of producers. Those interested in producing their own antisera may consult the methods given in section 14.

4.2 Presumptive identification

4.2.1 Bacitracin disc test

The high sensitivity of group A β-haemolytic streptococci to bacitracin is used as a screening method for differentiating between group A and non-group A streptococci (*51*). β-Haemolytic streptococcal strains showing zones of inhibition around special bacitracin sensitivity discs, containing 0.04 or 0.05 IU of bacitracin, are considered, presumptively, to be group A streptococci. Some investigators have also reported good success using discs containing 0.1 IU of bacitracin. However, others have reported that this concentration increased the number of non-group A strains exhibiting zones of inhibition (*52*). The concentrations of bacitracin mentioned produce at least some zone of inhibition in practically all strains of group A streptococci. On the other hand, the test results are not always specific for group A strains since a small fraction of group C and G streptococci display a similar sensitivity to bacitracin. The concentration is critical; discs containing higher concentrations of bacitracin should not be used.

Bacitracin discs can be produced in the laboratory but are also commercially available from several manufacturers. Instructions for use and for interpretation of results are always provided with the commercial discs and should be strictly followed.

The bacitracin test should be used only on pure cultures of streptococci, not on primary cultures on non-selective agar media. As with any antibiotic sensitivity test, many factors can influence the outcome; in particular, use of too light an inoculum or of blood agar plates that are too thin may result in oversized zones of inhibition.

4.2.2 PYR test

The PYR test is another method for presumptive identification of *S. pyogenes* (*53, 54*), which relies on the ability of organisms to hydrolyse L-pyrrolidonyl β-naphthylamide or L-pyroglutamic acid β-naphthylamide (PYR). Approximately 98% of group A streptococci appear to be PYR-positive. Although many bacteria of other Gram-positive, catalase-negative genera (i.e. *Enterococcus*, *Aerococcus* and *Gemella*) and species (*Lactococcus lactis*) are also PYR-positive, they are unlikely to be confused with group A streptococci because of their different morphology. Only haemolytic enterococcal strains could present problems, especially if grown on horse blood agar where haemolysis is more readily expressed than on sheep blood agar. It has also been reported that *Streptococcus suis* strains and at least one isolate of *S. equisimilis* (group C) are PYR-positive (*55*). Other investigators have found some human isolates of both group C and group G streptococci to be PYR-positive (A. Efstratiou and R. George, personal communication). Because of the wide range of PYR-positive non-streptococcal species, only pure cultures of streptococci should be tested.

There are several versions of the PYR test, including an agar medium containing PYR (*54*), PYR broth composed of Todd–Hewitt broth with PYR, and paper discs or filter-paper strips impregnated with PYR (*55*). The commercially available tests are nearly all of equal specificity and sensitivity, and any of them can be used with good results.

4.3 Group-antigen extraction methods

4.3.1 Fuller's method (formamide extraction) (*56*)

Equipment and supplies

— Centrifuge
— Oil-bath heater adjustable to 160 °C

Reagents

— Todd–Hewitt broth culture of the strain to be extracted, incubated for 17–24 hours at 37 °C
— Formamide
— Acid alcohol (1 ml of 36% hydrochloric acid, 99 ml of 95% ethanol)
— Acetone
— Saline, 0.85%
— Phenol red pH indicator solution. Dissolve 0.1 g phenol red in 28 ml sodium hydroxide, 0.01 mol/l, and adjust the volume to 250 ml with distilled water.
— Sodium hydroxide, 0.2 mol/l

Method

1. Centrifuge (approximately 1500 *g* for 30 minutes) 5 ml of the Todd–Hewitt broth culture and discard the supernatant.
2. Resuspend the pellet in 0.1 ml of formamide and mix well.
3. Heat at 160 °C in the oil-bath for 10 minutes or until almost completely dissolved.
4. Cool, add 0.25 ml of acid alcohol and shake.
5. Centrifuge (at least 650 *g* for 30 minutes) to remove the precipitate and collect the supernatant.
6. Add 0.5–1.0 ml of acetone (the amount depends on formation of precipitate) to the clear supernatant fluid.
7. Centrifuge (at least 650 *g* for 30 minutes) to collect the precipitate (group-specific polysaccharide).
8. Discard the supernatant and dissolve the precipitate in 0.3–0.4 ml of saline.
9. Add 1 drop of phenol red solution and adjust the pH to 7.2 with sodium hydroxide, 0.2 mol/l.

Comment

- Cross-reactions may occur if the extract is too alkaline.

4.3.2 Lancefield method (hydrochloric acid extraction) (*57*)

Equipment and supplies

— Centrifuge
— Water-bath, at 100 °C

Reagents

— Todd–Hewitt broth culture of the strain to be extracted, incubated overnight at 35–37 °C

— Hydrochloric acid, 0.2 mol/l (may be prepared in 0.85% saline; see comments below)
— Sodium hydroxide, 1.0 mol/l
— Sodium hydroxide, 0.2 mol/l
— Phenol red pH indicator solution (see section 4.3.1)

Method

1. Centrifuge (650–1500 *g* for 30 minutes) 30–40 ml of the Todd–Hewitt broth culture and discard the supernatant. If a firm, secure pellet is not obtained, the higher speed or longer centrifuging times will be required.
2. Resuspend the pellet in 0.35 ml of hydrochloric acid.
3. Heat this suspension in a boiling water-bath (100 °C) for 10 minutes.
4. Cool the suspension to room temperature and add one drop of phenol red solution.
5. Adjust the pH initially with sodium hydroxide, 1 mol/l. As the end-point is approached, use sodium hydroxide, 0.2 mol/l, for the final adjustment.
6. Centrifuge as above and collect the supernatant fluid (Lancefield extract).

Comments

- The Lancefield hot acid extract contains not only the group-specific antigen, but also type-specific protein antigens of group A (and group B) streptococci. Other nonspecific protein and non-protein antigens may also be present.
- Alternatively, 10 ml of Todd–Hewitt broth culture or colonies harvested from a quarter of a blood agar plate culture may provide sufficient antigen for group determination.
- In some instances growth in larger volumes (e.g. 80 ml extracted with 0.5 ml HCl) may prove useful.
- Lancefield used hydrochloric acid, 0.2 mol/l, prepared in 0.85% saline for the acid extraction of streptococci, and many laboratories continue to use this formulation.

4.3.3 El Kholy method (nitrous acid extraction) (*58*)

Equipment and supplies

— Centrifuge
— Pipettes to measure 0.12 and 0.06 ml (Method 1). Pasteur pipettes or microlitre pipette and tips (Method 2)
— Small culture tubes (12 × 75 mm) with stoppers, for preparation and storage of extracts

Reagents

— Todd–Hewitt broth culture of the strain to be extracted, incubated overnight at 35–37 °C
— Sodium nitrite, 4 mol/l
— Glacial acetic acid
— Phenol red pH indicator solution (see section 4.3.1)
— Sodium hydroxide, 6 mol/l
— Saline, 0.85% (Method 2)

Method 1

1. Centrifuge (1500 *g*, 30 minutes) 5 ml of the Todd–Hewitt culture and discard the supernatant.

2. Add 0.12 ml of sodium nitrite and 0.06 ml of glacial acetic acid to the pellet and mix thoroughly.
3. Incubate at room temperature for 15 minutes.
4. Add 1 drop of phenol red and neutralize the suspension to pH 7.0 with sodium hydroxide.
5. Centrifuge as above and collect the supernatant fluid (nitrous acid extract).

Method 2

1. Centrifuge 5 ml of the Todd–Hewitt culture and discard the supernatant.
2. Add 1 drop (approximately 40 µl) of saline and mix thoroughly.
3. Add 2 drops (approximately 80 µl) of sodium nitrite and 1 drop (approximately 15 µl) of glacial acetic acid and mix thoroughly.
4. Incubate at room temperature (20–22 °C) for 15–30 minutes. Do not seal the tube tightly at this stage since gases are being generated that could create dangerous pressure.
5. Add a small amount of phenol red (approximately 20 µl) and neutralize the suspension to pH 7.0 with NaOH.
6. Centrifuge (at least 650 *g* for 30 minutes), and collect the supernatant fluid (nitrous acid extract).

Comment

- An alternative procedure for extraction of the carbohydrate by nitrous acid involves scraping off the overnight culture from one-half or one-quarter of a blood agar plate, resuspending the streptococci either in a mixture of 0.12 ml of sodium nitrite, 4 mol/l, and 0.06 ml of glacial acetic acid (see Method 1) or in 1 drop of saline (see Method 2) and then preparing the extract as described above.

4.3.4 Rantz and Randall method (extraction by autoclaving) (*59*)

Equipment and supplies

— Centrifuge
— Autoclave
— Pipettes to measure 0.5 ml
— Small culture tubes (12 × 75 mm) with stoppers, for storage of extracts

Reagents

— Todd–Hewitt broth culture of the strain to be extracted, incubated overnight at 37 °C
— Saline
— Thymol blue or phenol red pH indicator solution (see sections 4.3.1 and 7.1)
— Sodium hydroxide, 1 mol/l

Method

1. Centrifuge (1500 *g*, 30 minutes) 40 ml of the Todd–Hewitt broth culture and discard the supernatant.
2. Resuspend the pellet in 0.5 ml of saline.
3. Autoclave the suspension for 20 minutes at 121 °C.

4. Centrifuge the autoclaved suspension as above and collect the supernatant extract.
5. Neutralize the clear supernatant (pH 7.0–7.2) with sodium hydroxide, using thymol blue or phenol red solution as indicator.

4.4 Comparison of group-antigen extraction methods

The Lancefield hot acid extraction method is ordinarily quite satisfactory, and it has the advantage that a single extract can be used for group identification, M typing and opacity-factor detection. However, since other streptococcal antigens are also extracted by this method it is possible for cross-reactions that are not group-specific to occur. This can be avoided if carefully tested and absorbed antisera are used. The formamide extraction method is, on the whole, least likely to give cross-reactions since protein antigens, which are frequently responsible for these nonspecific reactions, are destroyed. It has the disadvantage, however, that the heat of the extraction may destroy certain less resistant group-specific antigens (e.g. group O). Also, if M typing is to be performed, a separate Lancefield extract must be prepared. The method of Rantz and Randall is very simple, as is the El Kholy technique using nitrous acid. No cross-reactions with heterologous sera have been observed. There is good agreement between the nitrous acid and the hot hydrochloric acid extraction procedures. Other possible methods include extraction with *Streptomyces albus* enzyme and with pronase B, but these are not discussed here since they are not widely used. Pronase B has been used in some rapid antigen detection tests but has been found to be reliable only with group A strains (*36*).

4.5 Methods for group-antigen detection

4.5.1 Precipitation in capillary tubes (*60*)

Equipment and supplies

— Glass capillary tubes, inside diameter 0.5–1.2 mm, length 5–8 cm (see Comments)
— Rack with non-hardening modelling clay
— Tissue or other soft absorbent material for wiping the capillary tubes

Reagents

— Group-specific antisera
— Streptococcal extract containing group antigen
— Reference group A polysaccharide antigen

Method

1. Dip a tube into the group-specific antiserum and draw up a 1-cm column by capillary action.
2. Wipe the end of the capillary tube and dip it into the streptococcal extract; draw in a 1-cm column of extract (total length of column, 2 cm).
3. Insert the capillary tube into the rack using the modelling clay, with the antiserum above the extract.
4. Read after 5 minutes at room temperature. Disregard reactions that occur after 30 minutes.

Comments

- As a routine, all extracts should be tested against antisera for groups A, C and G.
- If potent antisera are used, capillary tubes with an inside diameter of 0.5–0.9 mm produce satisfactory reactions, even if significantly less antiserum is used (strong precipitin reactions can be produced with an antiserum layer of only 0.5 cm or less). Weaker antisera may require larger capillaries (inside diameter 0.9–1.2 mm).
- Utmost cleanliness must be maintained when handling capillary tubes. Finger marks or other contamination on the surface of the tube may mask weak positive reactions.
- The precipitation test in capillary tubes can also be performed as a ring test. The procedure is the same as that described above, except that the capillary is inverted before it is inserted into the rack so that the antiserum is below the extract.

The capillary precipitin test is illustrated in Fig. 6. Note the precipitate in capillary tube A as compared with the negative reaction obtained with the heterologous extract in tube B.

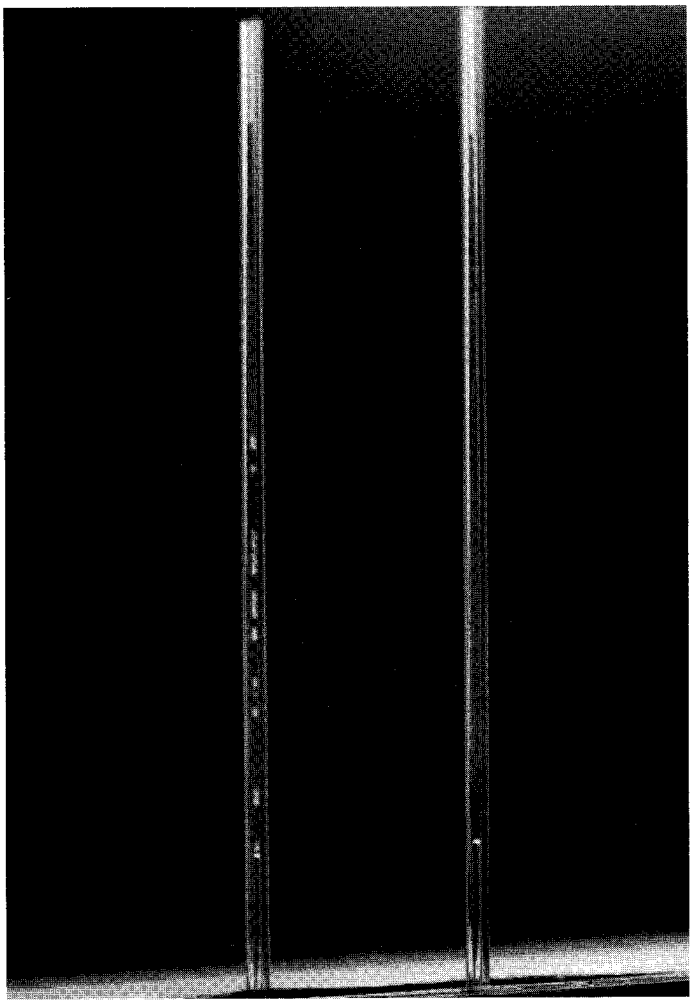

Fig. 6. The capillary precipitin test

4.5.2 Ring precipitin test (*61*)

Equipment and supplies

— Capillary tubes, conically narrowed and sealed at the end. The inside diameter of the wider part is about 0.5 cm, and the total length is 3–4 cm. These can easily be prepared by cutting off the bottom of a Pasteur pipette (see Fig. 7)
— Rack with non-hardening modelling clay
— Pasteur pipettes

Reagents

— Group-specific antisera
— Streptococcal extract containing group antigen
— Reference group A polysaccharide antigen

Method

1. Insert a capillary tube in the rack using the modelling clay.
2. Use a Pasteur pipette to deliver group-specific antiserum up to the middle of the tapered part of the capillary (see Fig. 7).
3. Overlay the extract on top of the antiserum, taking care that the two liquids do not mix.
4. Read against a dark background within 2–3 minutes. If the reaction is positive, a precipitation ring appears at the interface between the extract and the antiserum. Reactions should not be accepted as positive when they take more than 5 minutes to appear.

Comments

- All extracts should be tested against antisera for groups A, C and G.

The ring precipitin test is illustrated in Fig. 7. Note the thin layer of precipitate that has formed in tube A at the interface between the antiserum in the lower layer and the extract in the upper layer. Tube B contains the heterologous negative extract control.

4.5.3 Precipitation in agar gel (Ouchterlony double-diffusion test) (*62*)

The materials and method for preparation of agar gel slides are given in section 7.2. For group identification, use Pasteur pipettes to fill the central well with group-specific antiserum and the peripheral wells with extracts of the strains to be classified. In each pattern of wells there should be at least one peripheral well filled with a reference control extract of the group being tested. Incubate at 4 °C for 48 hours or at room temperature overnight in a moist chamber. Read and record the precipitation bands. All extracts should be tested against antisera for groups A, C and G.

4.6 Rapid grouping methods

Many new methods that have been developed for grouping streptococci allow reliable group identification from both liquid (Todd–Hewitt broth) and solid (blood agar) culture media. Compared with classical extraction and

Fig. 7. The ring precipitin test

serological testing methods, the time required is reduced from several hours to less than 1 hour. Several commonly used methods are based on slide agglutination for the detection of group antigen. These tests use group-specific hyperimmune rabbit antisera or, preferably, isolated immunoglobulin G bound either to latex particles (latex agglutination) (*63*) or to a treated cell suspension of a protein A-carrying *Staphylococcus aureus* strain (coagglutination) (*64*). Mixing an extract containing group antigen with these immunoglobulin-coated particles will yield an agglutination that is visible to the naked eye.

Tests that allow direct identification of group A streptococci from the primary throat swab are also commonly used and reduce the time necessary for diagnosis from 48 hours to 10 minutes or less. Effective therapy can thus be

started immediately. The direct tests consist of two steps: group polysaccharide extraction directly from a swab; and group A streptococcal polysaccharide detection. Various immunodetection methods can be used, including latex agglutination, coagglutination and ELISA. A number of tests based on these principles are commercially available. Test kits must contain all items necessary for testing, i.e. swabs, extraction tubes, chemicals for group polysaccharide extraction, the group-specific immunoreagent and positive and negative controls. Since the sensitivity of rapid tests is lower than that of a properly performed standard culture, all negative results of rapid tests in patients suspected of having a streptococcal infection must be checked by culture (*65*).

5. Characterization of group A streptococci

5.1 Serological methods for strain characterization

The classification of group A streptococci into specific serotypes is based upon the detection and identification of cell-surface protein antigens referred to as T, R and M proteins. Of these, the M protein is considered the most important: it is not only a highly specific antigenic marker, but also a major virulence factor of these organisms. Protective immunity against infection is essentially type-specific and depends on the formation of antibodies to M protein. Certain M types produce and liberate another type-specific substance called opacity factor. Since the antigenic specificity of opacity factor parallels that of M protein, opacity factor typing is considered equivalent to direct identification of M antigen. Most group A streptococcal strains freshly isolated from human sources possess sufficient type-specific antigen to be typable provided that they are properly stored and handled in the laboratory, that corresponding antisera are available, and that reliable, sensitive typing methods are used. M-type identification is most commonly performed using an immunoprecipitation test (see section 7), but it can also be done — with much more difficulty — using the indirect bactericidal test, as described in section 13.

Use of M typing has opened the way to a better understanding of the bacteriology, immunology and epidemiology of streptococcal infections. Tracing the changing type distribution in population groups is important for the development of a streptococcal vaccine based on M proteins. M typing is also of great importance in studies concerned with the nephritogenicity or rheumatogenicity of specific strains of group A streptococci, as well as in surveillance of unusual infections and studies of the epidemiology of streptococcal infection outbreaks.

Strains for which M typing is not possible can usually be characterized by means of antisera raised against T-protein antigens. In group A streptococci, these have no known relationship to virulence or protection. There are fewer recognized T antigens than there are M antigens, and they are less specific markers than M protein. A single T pattern may be found in strains of different M types, and strains of the same M type may carry one or another T-protein antigen; for this reason the term "T pattern" is preferred to T type. Although the T pattern provides useful information, it is not an M-type specific marker. Analogous T-protein antigens are also produced by strains of other serological groups, especially groups C and G.

R-protein antigens occur in some strains of M types 2, 3, 28, 33, 43 and 48, as well as in strains of some other serological groups (B, C, G, L) (*66*). They may be responsible for cross-reactions in typing or grouping. Four R antigens have been described and designated R1 to R4; specific strains may carry only one or a combination of these (*66*). R proteins are not associated with virulence, and the antibodies they elicit have no known influence on human immunity to group A streptococci. Because R proteins are trypsin-resistant, trypsin digestion of an M-protein extract will have no effect on R-protein antigen–antibody reactions. This property allows differentiation of R-protein from M-protein reactions.

5.2 Non-serological methods for strain characterization

Streptococcus pyogenes isolates can also be characterized using non-serological methods. These were developed, in part, in response to the difficulties often encountered in producing the large pool of type-specific antisera required for streptococcal serotyping. Apart from obviating the need for antiserum production, non-serological methods have the added advantage that they can often be used to characterize and/or define relationships between strains that cannot be typed using conventional techniques. These methods also often provide a means of identifying specific strains, or subtypes, within existing serologically identified M types.

5.2.1 Phage typing

One method developed for subtyping group A streptococci was based on the principles utilized in phage typing of staphylococci, and was originally used in epidemiological studies of M49 streptococci. It has permitted accurate discrimination of M49 strains isolated from different geographical regions as well as strains of this M type isolated from the same location in different years (*67*, *68*), proving useful in studies of the detailed epidemiology of this important nephritogenic M type.

5.2.2 Bacteriocin typing

Production of bacteriocin-like inhibitory substances by group A streptococci has been utilized in an alternative typing scheme (*69–71*). By testing a strain for inhibitory activity against a standard set of nine indicator strains, a type — referred to as the P type — can be established. This P type, made up of a three-digit number based on the specific strains inhibited, appears to be a stable strain identification marker. Although not M-type specific, this method has proved to be a useful epidemiological tool and, when combined with M typing, improves the discrimination of streptococcal strain subtypes.

5.2.3 Small-fragment DNA gel electrophoresis

Restriction fragment length polymorphism (RFLP) analysis of group A streptococcal genomic DNA has been shown to be a useful method for strain characterization. DNA is extracted from streptococcal cells and cleaved by using a restriction enzyme (or a combination of restriction enzymes), which cuts the DNA at many sites ("frequent cutting" enzymes). The DNA fragments are then separated by agarose gel electrophoresis. The resulting DNA "fingerprint" appears to be a stable strain marker and can be used to establish clonal relationships between strains (*72*). All strains can be characterized by this technique, although the large number of bands obtained may make interpretation difficult. Strains of the same M type often have identical or very similar restriction patterns, although pattern variation within M types can be significant. This technique has also been used successfully to identify epidemiologically important strain differences between isolates of the same M type (*73*).

5.2.4 Large-fragment DNA pulsed field gel electrophoresis

A modification of the small-fragment DNA fingerprinting method utilizes "rare cutting" restriction enzymes to cleave genomic DNA into fewer fragments than would be obtained using frequent cutting enzymes. This ad-

dresses one of the major difficulties encountered with conventional RFLP methodology, that of visually comparing and resolving the large number of DNA fragment bands obtained. Cutting the DNA into fewer (but larger) fragments results in electrophoresis banding patterns that are easier to interpret. Unfortunately, these large fragments cannot be analysed by standard gel electrophoresis but require the more expensive pulsed field gel electrophoresis (PFGE) equipment. It has been reported that individual M types give distinct PFGE patterns and that clonal variation within an M type can also be differentiated (*74*).

5.2.5 Ribotyping

A variation of the RFLP method has been described; it utilizes DNA restriction patterns of ribosomal RNA genes (ribotyping) to characterize group A streptococci. To date, most published reports have suggested that this method may be less effective than the standard total DNA RFLP method in discriminating subtle differences between group A streptococcal strains (*75–77*). Used alone, however, or in combination with RFLP analysis, this method can improve the discrimination of serotyping.

5.2.6 Oligonucleotide probes

A non-serological method for the identification of specific M proteins of group A streptococci using oligonucleotide probes has been described (*78*). Probes directed against the *N*-terminal region of specific M-protein genes were constructed to provide a highly specific typing system. In an evaluation of this system using probes to nine different M types tested blind against 116 strains with 10 different M types, sensitivity and specificity were 100%. Oligonucleotide probes are reported to be cheaper and easier to prepare than M-type antisera, and they can also be constructed to detect and identify subtle sequence diversity within specific M types (*78*).

5.2.7 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis of cell lysates has also been used for characterization of group A streptococcal strains (*79–81*). Isolates are tested for the presence and electrophoretic mobility of 12 different enzymes and assigned a number, referred to as the electrophoretic type, based on the activity and migration profiles obtained. All streptococcal strains tested could be typed by this method, and a strong correlation with M type was reported, although exceptions were observed (*81*).

5.2.8 Pyrolytic mass spectrometry

Pyrolytic mass spectrometry has been used as a tool in studying the epidemiology of nosocomial infections (*82, 83*); the method allowed *S. pyogenes* identification at the strain level and proved to be a rapid technique for use in studies of cross-infection. However, the equipment required is expensive and the computer analyses of collected data are complex. This method is therefore probably impractical for laboratories that do not already possess the necessary equipment and computer expertise.

5.2.9 M protein gene sequencing

Recent developments in molecular technology have made possible the routine use of DNA sequencing of specific genes as a tool in streptococcal strain

identification. Of particular interest is the ability to sequence the M protein (*emm*) gene. This procedure involves extraction of DNA from the streptococcal strain, amplification of the *emm* gene in the polymerase chain reaction (PCR) and sequencing of the amplified DNA. The resulting sequences permit not only identification of specific M types by matching with published sequences but also determination of variation within an M type and characterization of *emm* genes not previously described (*84–87*).

5.2.10 Random amplification of polymorphic DNA

Random amplification of polymorphic DNA (RAPD) analysis has proved to be a useful tool for genetic studies of plants, animals and microbes. This system potentially offers improved discrimination over methods that rely on phenotypic markers, since it allows comparison of entire genomes. In at least one study, RAPD was shown to be more sensitive than multilocus enzyme electrophoresis in differentiating closely related bacterial strains (*88*). DNA extracted from an organism is amplified in PCR using primers of less arbitrary sequences. While one advantage of this system is that no knowledge of the strain's DNA sequence is required in selecting primers, there is also no way to predict which primer sequences will be most effective in discriminating streptococcal strains. It may be necessary to test a large number of primers against many well characterized strains to determine which primers and primer combinations provide the greatest resolution. RAPD has been successfully used by several investigators to differentiate strains of *Streptococcus pyogenes* (*89–91*).

5.2.11 *Vir* typing

A recently described method for characterization of *S. pyogenes* strains utilized PCR amplification of the entire *vir* regulon, followed by restriction digestion of the amplified DNA product with *Hae*III. The cleaved DNA was then subjected to agarose gel electrophoresis. Resulting DNA bands were visualized by ethidium bromide fluorescence. The investigators found this method to be highly discriminatory and to yield unambiguous results (*91*).

6. Determination of T-protein agglutination patterns

6.1 Introduction

Identification of the T-protein agglutination pattern is usually the first step in the classification process for group A streptococci. Various methods for this procedure have been described in the literature (*61, 92–94*). The distribution of T-protein antigens, which are produced by most strains of group A streptococci, is not directly related to that of the M antigens; a single T antigen (or very closely related T antigens) may be shared among a number of M types, while strains of a given M type may have one or another totally unrelated T antigen. The following clusters of M types appear to have the same (or a few very closely related) T antigen(s):

- 4, 24, 26, 28, 29, 46
- 15, 17, 19, 23, 30, 47
- 5, 11, 12, 27, 44
- 3, 13, and types agglutinated by antiserum B3264
- 8, 25, and types agglutinated by antiserum Imp. 19
- 14, 49

(For distribution of T patterns "B3264" and "Imp. 19" among M types, see Table 1.) Strains belonging to the "cluster types" may be agglutinated by several of the corresponding monovalent anti-T sera.

Diffuse, stable suspensions of streptococci are required for slide agglutination. Granular, spontaneously agglutinating cultures must be treated with trypsin to render them suitable for the T-agglutination test. Streptococci are agglutinated by antisera containing homologous anti-T antibodies. Since diffuse suspensions are also very susceptible to other agglutinins not specific for T antigens, absorbed anti-T sera must be employed. Anti-T sera sets are composed of polyvalent and monovalent antisera. Each polyvalent antiserum, or pool, is a mixture of the given monovalent antisera. The set originally described consisted of the following (*61*):

Pool	*Monovalent antisera*
T	1, 3, 13, B3264
U	2, 4, 6, 28
W	5, 11, 12, 27, 44
X	8, 14, 25, Imp. 19
Y	15, 17, 22, 23, 47
Z	9, 18, 19, 30

Rabbit anti-T sera are commercially available. Although some monovalent antisera, especially types 15, 17, 47, 19 and 30, are frequently omitted from anti-T sera sets, these types would be expected to react with remaining types in their respective pools (*93*). One commercially available set has eliminated the Z pool and uses a combined Y pool consisting of monovalent anti-T sera 9, 22 and 23.

Table 1. The relationship between T-protein agglutination pattern, M type and opacity factor in group A streptococci

T-protein agglutination pattern	M type[a]	
	Opacity factor positive	Opacity factor negative
1	68	1
2	2	65
3, 13, B3264	13, 73, 77, 81	3, 33, 39, 41, 43, 52, 53, 56, 67, 69, 71, 72, 74
4, 28	4, 28, 48, 60, 63	24, 26, 29, 46
5, 11, 12, 27, 44	11, 22, 27, 44, 61, 62, 66, 76, 78	5, 12, 70
6	—	6
8, 25, Imp. 19	8, 25, 58, 59, 75, 79	31, 55, 57
9	9	—
14, 49	49	14, 51, 80
15, 23, 47	—	15, 17, 19, 23, 30, 47, 54
22	22	—

[a] M numbers not used: M7, group C; M10 = M12; M16, group G; M20, group C; M21, group C; M35 = M49; M45 = M24. For types M8, M27 and M44 see section 8.

6.2 Preparation of streptococcal suspension for agglutination

Equipment and supplies

— Streptococcal strains to be tested
— Water-bath at 37 °C
— Incubator at 30 °C
— Centrifuge
— Pasteur pipettes

Reagents

— Todd–Hewitt broth (use volumes of 5 ml; larger volumes of 8–10 ml may also be used)
— Trypsin solution, 5%: trypsin (2.5 g trypsin 1:250 per 50 ml phosphate-buffered saline, pH 7.3; alternatively, prepare in Sorensen's phosphate buffer, pH 8.2). Membrane-filter to sterilize and store at –20 °C.
— Phenol red pH indicator solution. Dissolve 0.1 g of phenol red in 28 ml sodium hydroxide, 0.01 mol/l, and adjust volume to 250 ml with distilled water.
— Sodium hydroxide, 0.2 mol/l

Method

1. Inoculate the strain to be tested in 5–10 ml of Todd–Hewitt broth and incubate overnight at 30 °C.

2. Centrifuge (650–1500*g*, 15–30 minutes) and draw off all but 0.3–0.5 ml of the supernatant (depending on the cell volume) and resuspend the bacteria in the remaining supernatant.
3. Add one drop of trypsin and one drop of phenol red solution.
4. Adjust the pH to 7.8–8.0 using sodium hydroxide, 0.2 mol/l (violet colour). Alternatively, a slightly higher pH of 8.0–8.2 may be used.
5. Place the suspension in a water-bath at 37 °C for 1 hour.
6. Before testing by agglutination, bring the pH to 7.0–7.2. Alternatively, many researchers have good success testing the cell suspension at the digestion pH of 8.0–8.2 without neutralization.

Comments

- If the initial growth of the culture is insufficient, prolong the incubation for another 24 hours.
- For certain strains, growth at room temperature (approximately 20–22 °C) will give better T agglutination results.
- Most strains require trypsinization for successful testing. Occasionally strains are encountered that give acceptable results without use of trypsin.
- Since different lots of trypsin may vary in potency, it may be advisable to test the proteolytic activity of the 5% trypsin solution. Prepare serial dilutions of the working trypsin solution, and place drops of each dilution on exposed photographic film (used X-ray film works well). Incubate for 1 hour at 37 °C in a moist chamber. The titre of the trypsin is the highest dilution producing a clear area. A 10% concentration of trypsin 1:250 is reported to have a titre of not less than 1:10000 (*94*). A 5% concentration of trypsin 1:250 with a titre in excess of 1:2000 should be satisfactory for routine testing.
- Good success with considerable saving in time has been reported using a method whereby the strains are grown in Todd–Hewitt broth containing a final concentration of 0.6% trypsin (1:250). Incubation conditions and times are as described above. After growth in trypsin broth the culture is centrifuged as above and the streptococci resuspended in 0.3 ml of the supernatant broth. Agglutination testing is then performed directly without need for further digestion (*94*).

6.3 Performing the agglutination test

Equipment and supplies

— Microscope slides, 25 × 75 mm
— Wire loop(s) approximately 2–3 mm in diameter (if highly potent sera are used, smaller loops may be satisfactory)
— Water-bath at 50 °C

Reagents

— Trypsinized cell suspension prepared as described in section 6.2
— Polyvalent and monovalent anti-T sera

Method

1. Place five or six evenly spaced drops of the trypsinized cell suspension on a microscope slide (one drop for each polyvalent antiserum (pool) in the test kit being used).

2. Using the wire loop, add one drop of each of the polyvalent antisera to a corresponding drop of the strain suspension.
3. Rock the slide to mix.
4. Evaluate the reaction within 2 minutes. Immediate agglutination is marked as + + + or 3+, strong reaction within 1 minute as + + or 2+, and weak but distinct agglutination within 1–2 minutes as + or 1+.
5. If the suspension is agglutinated by only one polyvalent antiserum (pool), test the suspension in the same way with the corresponding monovalent antisera which compose that pool.
6. Agglutination by more than a single monovalent anti-T serum commonly occurs only within the clusters of types having common T antigen(s) (see section 6.1), but there are exceptions.
7. If the suspension does not agglutinate with any of the polyvalent or monovalent antisera, prepare a fresh culture and repeat the procedure using a shorter trypsin incubation time or even omitting trypsin digestion.
8. If the suspension is granular, or if agglutination occurs with multiple pools or with types other than the clusters described above, trypsinization of the suspension should be repeated for 1 hour with one more drop of trypsin at pH 7.8–8.0. The test should then be repeated as described above.
9. If the suspension still gives nonspecific agglutination, digest it again with an additional drop of trypsin in a water-bath at 50 °C for 10 minutes. If this is unsuccessful, further digestion is rarely useful, and the test should be repeated with a freshly prepared culture.

6.4 Evaluation of results

Most group A strains can be characterized by T agglutination. Although typing by M precipitation or opacity factor neutralization results in a reaction with only a single antiserum, in the T agglutination test a clear agglutination of a strain by several monovalent anti-T sera is often valid. Complex agglutination patterns such as 3/13/B3264, or 4/28 or 5/12/27 are quite common.

Some strains give a clear-cut agglutination with only a single monovalent anti-T serum: T1, T6, T9 and T22 are the most frequent examples. Such agglutination results often, but not always, correspond with the M types of the strains. These apparently unequivocal findings cannot, therefore, be safely accepted as equivalent to M-typing results.

The discrepancies observed are due not only to the lack of correlation in the distribution of T and M antigens already mentioned, but also to the fact that agglutination is not exclusively dependent on the reaction between T antigen and anti-T antibodies. No available set of anti-T sera covers all the existing T antigens. In absorbed anti-T sera, prepared by immunization with whole-cell vaccines, significant remnants of unrelated antibodies are to be expected. Indeed, most strains are agglutinated by several, if not all, polyvalent anti-T sera before trypsinization. Thus, in the process of testing, some of the remaining non-T antigens may agglutinate; if these reactions are strong enough and otherwise plausible, they may erroneously be accepted as the true T patterns. With conventional sets of anti-T sera, the risk of such mistakes increases with the rising prevalence in the population of strains with unusual T antigens or no T antigens. The reproducibility rate of T-agglutination patterns is thus lower than that of M or opacity factor typing.

Nevertheless, the technically uncomplicated T-protein agglutination test has been widely used for serological differentiation of group A strains in those laboratories without access to the more specific M and opacity factor typing tests. Generally, T-agglutination patterns are of much greater use in situations

where the number of types involved is limited, such as epidemiological analysis of localized outbreaks of streptococcal diseases. Even in this sort of situation, however, dependence on this characteristic alone may be misleading. Reliance on T-agglutination patterns alone from surveillance studies covering large population groups over several years may also lead to misinterpretation.

In summary, T-protein agglutination patterns cannot specifically identify types of group A streptococci or their relationship to virulence and protective immunity as defined by the M proteins. Similarly, the proposed broader interpretation of agglutination patterns in terms of a subdivision of group A streptococci into subgroups of certain M types may occasionally be grossly misleading. However, knowledge of the relationship between T and M antigens and opacity factor is extremely useful for the laboratory in the process of typing, since it reduces or eliminates the need to test strains with all M or opacity factor typing sera. These relationships are summarized in Table 1. A review of the correlation of T-protein agglutination pattern with M type has also been published (*95*).

7. Serotyping by M-protein precipitation

The precipitation reaction between M antigen and type-specific antiserum is employed for the identification of M types. Rabbit antisera are used.

7.1 Preparation of Lancefield hot hydrochloric acid M-antigen extract (96)

Equipment and supplies

— Incubator at 37 °C
— Centrifuge
— pH meter, pH paper or thymol blue pH indicator solution (dissolve 0.1 g of thymol blue in 4.3 ml of sodium hydroxide, 0.05 mol/l, and adjust the volume to 100 ml with distilled water)
— Water-bath at 100 °C (boiling)
— Pipettes to measure 0.4 ml
— Pasteur pipettes
— Small culture tubes (12 × 75 mm) with stoppers, for storage of extracts

Reagents

— Blood agar culture with isolated colonies or broth culture of streptococci
— Todd–Hewitt broth containing an additional 1–2% neopeptone
— Hydrochloric acid, 0.2 mol/l (may be prepared in 0.85% saline; see comments below)
— Sodium hydroxide, 0.2 mol/l
— Phenol red pH indicator solution. Dissolve 0.1 g of phenol red in 28 ml of sodium hydroxide, 0.01 mol/l, and adjust volume to 250 ml with distilled water.
— Thiomersal[1], 0.02%, or sodium azide preservative solution, 0.1%

Method

1. Inoculate several isolated colonies or 1 ml of Todd–Hewitt broth culture in 80 ml of prepared Todd–Hewitt/neopeptone broth.
2. Incubate for 16–18 hours at 37 °C. Plate a loopful on blood agar to test for purity.
3. Harvest the bacteria by centrifugation at 650–1500 *g* for 30 minutes and discard the supernatant (see comments below).
4. Add 0.4 ml hydrochloric acid to the pellet and mix thoroughly. Adjust the pH to 2.0–2.2 (see comments below).
5. Place the suspension in a boiling water-bath for 10 minutes.
6. Cool the suspension to room temperature.
7. Add one drop of phenol red solution and neutralize with sodium hydroxide.
8. Centrifuge the suspension as above. The supernatant fluid is the M-protein extract (hydrochloric acid extract). Store at 4 °C with sodium azide or thiomersal (1 drop/ml) as a preservative, or frozen at –20 °C.

[1] United States Pharmacopeia terminology: thimerosal.

Comments

- Todd–Hewitt broth with neopeptone is necessary to block proteinases that digest M protein, and to stimulate the synthesis of M protein (*17–19*).
- After centrifuging the initial culture, keep a small quantity of supernatant (about 2 ml) for serum opacity reaction testing (see section 8). Preserve with 2 drops of thiomersal at 4 °C.
- The pH of the cell/acid suspension for extraction *must* be about 2.0–2.2, not less; in a more acidic medium, hydrolysis and destruction of M protein occur. Control the pH of the streptococcal suspension in hydrochloric acid by means of a pH meter, pH paper or thymol blue solution (mix one drop of the suspension with one drop of thymol blue on a white slide — the colour must be only light pink, not red).
- Lancefield prepared acid extracts using hydrochloric acid, 0.2 mol/l, in 0.85% saline, and many laboratories continue to use this formulation.

7.2 M-antigen detection in agar gel (Ouchterlony double-diffusion test) (97, 98)

Equipment and supplies

— Steam-bath or boiling water-bath to melt agar
— Microscope slides, 25 × 75 mm
— Level tray for pouring slides
— Punches for cutting wells 3 mm in diameter
— Pasteur pipettes

Reagents

— Noble agar
— Phosphate buffered saline, pH 7.0
— Sodium azide
— M-protein hydrochloric acid extracts
— M-type specific antisera

Method

1. Prepare a solution of noble agar in phosphate-buffered saline, 0.01 mol/l, pH 7.0, and add sodium azide to give a final concentration of 0.01%.
2. Melt the agar by steaming or heating in boiling water. The agar can be used immediately or stored in aliquots at 4 °C until needed.
3. Cool the agar to 50 °C. Place the glass microscope slides on the level tray and pour 3 ml of agar onto each; use of 2 ml of agar per slide has also been successful.
4. After the agar has solidified, store the slides in a moist chamber at 4 °C.
5. Immediately before use, cut two sets of wells in each slide, each set consisting of 6 or 8 peripheral wells and a central well. The diameter of the wells should be 3 mm, and the distance from the centre of the central well to the centre of the peripheral wells 7.5 mm.
6. Using Pasteur pipettes, place the anti-M sera in the central well, and the M-protein hydrochloric acid extracts in the peripheral wells. Include the hydrochloric acid extract of a reference homologous type with each antiserum.
7. Incubate in a moist chamber at 4 °C for 48 hours or at room temperature overnight.
8. Read and record the results.

Comments

- The M type is identified if the extract of the strain gives a clear precipitation reaction with one absorbed, M-type-specific antiserum only.
- Unabsorbed anti-M sera can be used, which is one advantage of the agar gel precipitin technique. However, the antisera selected for typing should not elicit bands due to group A carbohydrate or to other non-type-specific cross-reacting antigens, including R proteins.
- The type-specific and non-type-specific bands can be differentiated by comparing reactions with that of the reference homologous hydrochloric acid extract. The band formed by the test extract must form a line of identity with the reference M-type extract.
- If R-protein bands are suspected, especially with antisera of types 28 and 48, the test should be repeated with the same hydrochloric acid extract after trypsinization (M protein is destroyed, R protein persists). In certain instances, absorbed anti-M sera, if available, are used.
- In the macroimmunodiffusion test, hydrochloric acid extracts often give a type-specific reaction but with multiple bands. This may be because the extract contains multiple fragments of M protein of quite different size as a result of a strong and nonspecific acid hydrolysis. These multiple bands usually, but not always, coalesce to form only a single band in the microimmunodiffusion test.
- Negative results (absence of precipitation bands) do not exclude the possibility that the strain belongs to a type for which a potent antiserum was included in the test. The reason may be inadequate quality of the hydrochloric acid extract, insufficient quantity of extract in the well, or some other reason as yet not understood. Sometimes an extract that gives a negative reaction initially will give a positive reaction when the test is repeated.
- The risk of possible misinterpretation of findings in a microimmunodiffusion test may be reduced by comparing them with the results of serum opacity reaction testing and, to a certain extent, also with the T-protein agglutination pattern of the strain.

An Ouchterlony double-diffusion test is illustrated in Fig. 8. In this test the purpose was to compare the reactions of several antisera with a single M1 hydrochloric acid extract. The extract was therefore placed in the centre well and the antisera in the peripheral wells. The results clearly show that the unabsorbed antiserum gives two precipitin bands with the M1 extract. The outer band forms a line of identity with a reference group A antiserum.

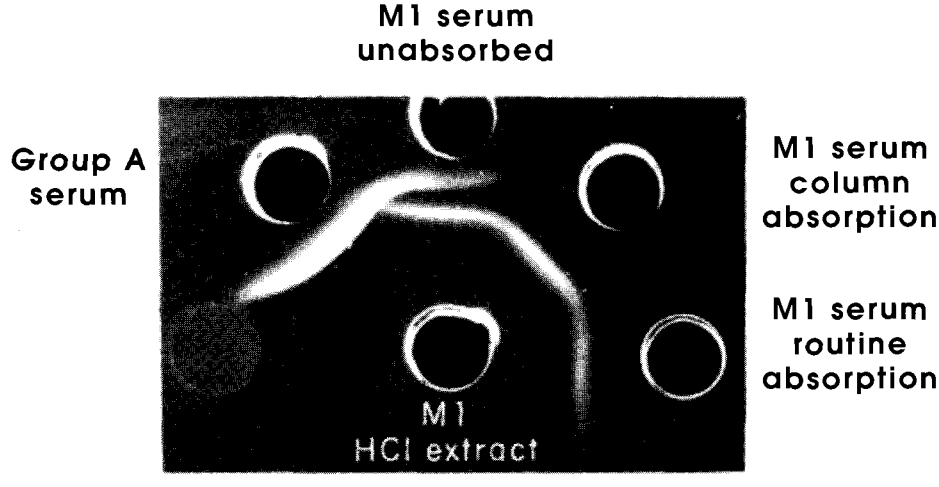

Fig. 8. M-antigen detection in the Ouchterlony double-diffusion test

The two absorbed antisera have had the group antibody successfully removed and contain only M1 type-specific antibody.

7.3 Capillary precipitin test (99)

The original precipitin method using capillary tubes needs highly potent and well absorbed anti-M sera. Preparation of these absorbed antisera is a very demanding laboratory procedure with uncertain results, and the sera do not always keep well. The equipment and performance of the test are the same as described in section 4.5.1 except for incubation conditions: the capillaries are incubated for 2 hours at 37 °C (first reading) and then overnight at 4 °C (final reading).

The Ouchterlony double-diffusion method is faster and easier, and absorbed antisera are not, for the most part, necessary. Good agreement is observed between M typing by the capillary method and by the double-diffusion method.

8. Serotyping using the serum opacity reaction

8.1 Introduction

Strains belonging to certain M types (see below) produce opacity in mammalian sera as a result of the action of an enzyme, referred to as serum opacity factor (OF), on high-density lipoproteins (HDL) (*100–103*). There are many antigenic variants of OF and their antigenic specificity corresponds with that of the M antigen of the producing strain (*104, 105*). Production or non-production of OF is a constant characteristic of each M type (*106, 107*). Thus, M types may be divided into OF-positive (OF+) and OF-negative (OF–) serotypes. All strains belonging to an OF+ M type produce serum opacity factor, although only M-positive strains secrete this enzyme during growth; M-negative variants appear to have the OF enzyme bound to the cell membrane (*105*). Based on current knowledge, it is highly unlikely that a strain that is found to be a producer of OF would belong to any of the M types recognized as OF-negative.

Opacity factor can be detected in broth culture supernatants, in hydrochloric acid extracts, in areas surrounding colonies on pour plates containing serum, and in sodium dodecyl sulfate or deoxycholate extracts of streptococci. OF typing is based on specific inhibition of the serum opacity reaction by antiserum containing anti-OF antibody; rabbit or guinea pig antiserum is commonly used. Opacity factor appears to be highly antigenic in humans and therefore, with extreme caution, it is also possible to use human sera containing anti-OF antibodies in OF-inhibition typing tests. Human sera often contain antibodies to serotypes commonly found in the population, including serotypes not previously identified. The sera are easy to obtain and are immediately available for use. However, in contrast to hyperimmune animal sera, individual human sera may, and frequently do, contain anti-OF antibodies to more than one serotype (*108–111*). Hence, it is *never* possible to rely on inhibition by a single human serum to define the M/OF type of an unknown strain. Probability of error when using human sera for OF typing can be reduced by use of multiple sera, each known to contain antibody to the serotype being screened.

The following M types have been recognized as OF+: 2, 4, 9, 11, 13, 22, 25, 28, 48, 49, 58, 59, 60, 61, 62, 63, 66, 68, 73, 75, 76, 77, 78, 79, 81. As yet there is no consensus for M types 8, 27 and 44, although these have been reported by some investigators, including the Minneapolis laboratory, as OF+. Additional OF+ types are being encountered among group A streptococci that cannot be typed with the available antisera.

The serum opacity reaction greatly enhances the possibilities for serotyping group A streptococcal strains. OF+ types are frequently those for which it is difficult to produce good anti-M sera. A combination of both serotyping methods, M precipitation and OF neutralization, is therefore very useful. OF results also help to check the accuracy of M-precipitation results. Neutralization of the opacity reaction by more than a single monospecific anti-OF serum is a very rare event; when it occurs, the test should be repeated with a newly prepared OF supernatant or extract, and re-evaluation of the sera being used should be considered. If none of the available anti-OF sera neutralizes

the serum opacity reaction, repeating the test with a lower concentration of the OF preparation may sometimes be successful.

8.2 Sources of opacity factor and opacity factor substrate

Overnight broth culture supernatants, hydrochloric acid extracts or sodium dodecyl sulfate extracts of OF+ strains are used as the source of OF. Horse or pig serum is generally used as the substrate (sterilized by filtration). It is recommended that this serum be kept in small aliquots at –20 °C. Each batch of serum should be tested for activity with known OF-producing streptococci since sera vary widely in their suitability for use in this test. Thiomersal is added to horse or pig sera and to all supernatants or extracts in a final concentration of 1:5000 (0.02 g/100 ml).

8.3 Agar method for detecting opacity factor (112)

Equipment and supplies

— Steam bath or boiling water-bath to melt agar
— Glass slides, 5 × 5 cm or 2.5 × 7.5 cm, or standard 10-cm Petri dishes
— Wire loop(s), approximately 4 mm in diameter
— Water-bath at 50 °C
— Level tray for pouring agar slides
— Pipettes to measure 1.0 ml
— Incubator (35–37 °C)
— Moist chamber

Reagents

— Pig or horse serum prescreened and selected to give good opacity reactions
— Culture supernatant, hydrochloric acid extract or other opacity factor extract to be tested
— Ion agar or purified agar
— Control supernatant or extract known to be OF+.

Method

1. Prepare 2% ion agar or purified agar in distilled water.
2. Melt the agar and cool to 50 °C.
3. Mix 1 ml of pig or horse serum at 50 °C with 1 ml of 2% agar. These are the quantities required for a slide measuring 5 × 5 cm. If a smaller slide or a Petri dish is used, the agar/serum volumes should be adjusted accordingly.
4. Pour the whole mixture evenly on a levelled glass slide or Petri dish that is thoroughly clean and free of grease.
5. Dry at 37 °C for 15 minutes.
6. Spot the supernatants or extracts on the agar surface using the wire loops.
7. Incubate the slides overnight at 37 °C in a moist chamber.
8. Read and record the results. A positive reaction is shown by the development of an opaque spot.

Comments

- The serum agar slides can be prepared in advance and stored in a moist chamber at 4 °C for up to 1 week.

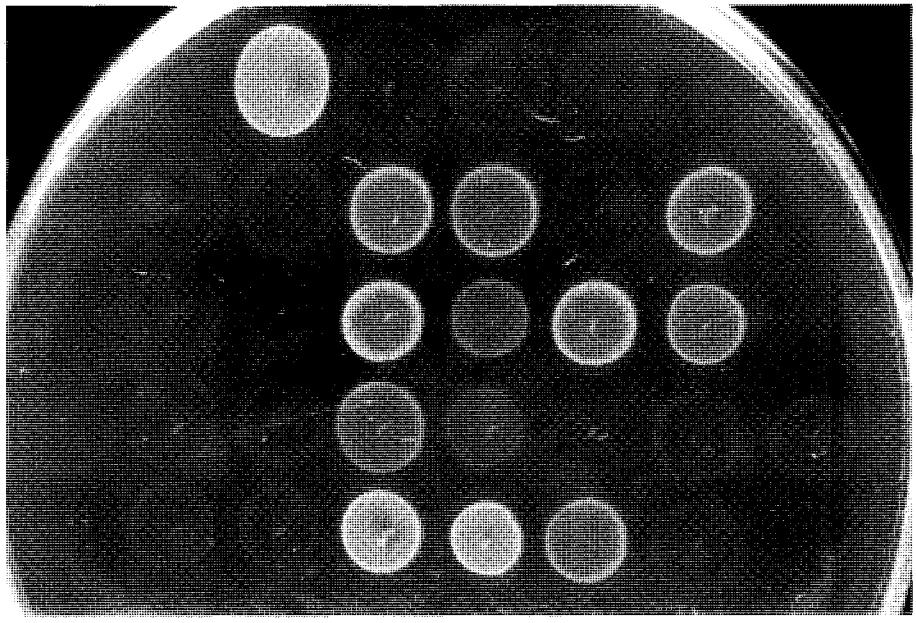

Fig. 9. The agar method for the detection of serum opacity factor

- Adjusting the pH of the horse or pig serum to 6.0 enhances the intensity of the opacity reaction.

Fig. 9 illustrates OF detection using the agar method in a Petri dish; OF preparations of different strains have been spotted on the agar surface. Positive opacity reactions of varying intensity are visible as opaque spots on the clear agar.

8.4 Agar method for opacity-factor inhibition serotyping (112)

8.4.1 Method 1

Equipment and supplies

— As described in section 8.3.

Reagents

— As described in section 8.3.
— Type-specific anti-OF sera

Method

1. Prepare the slides as described in section 8.3.
2. Mix 1 ml of pig or horse serum, 1 ml of 2% agar and 0.2 ml of culture supernatant or extract of the OF+ strain under test. These are the quantities required for a slide measuring 5 × 5 cm. If a smaller slide or a Petri dish is used, the volumes should be adjusted accordingly.
3. Pour the mixture onto a glass slide or Petri dish. Let the agar gel dry for 15 minutes at 35–37 °C and immediately spot anti-OF sera onto the agar surface using a wire loop. One glass slide suffices for 16 antisera.
4. Incubate overnight at 35–37 °C in a moist chamber.

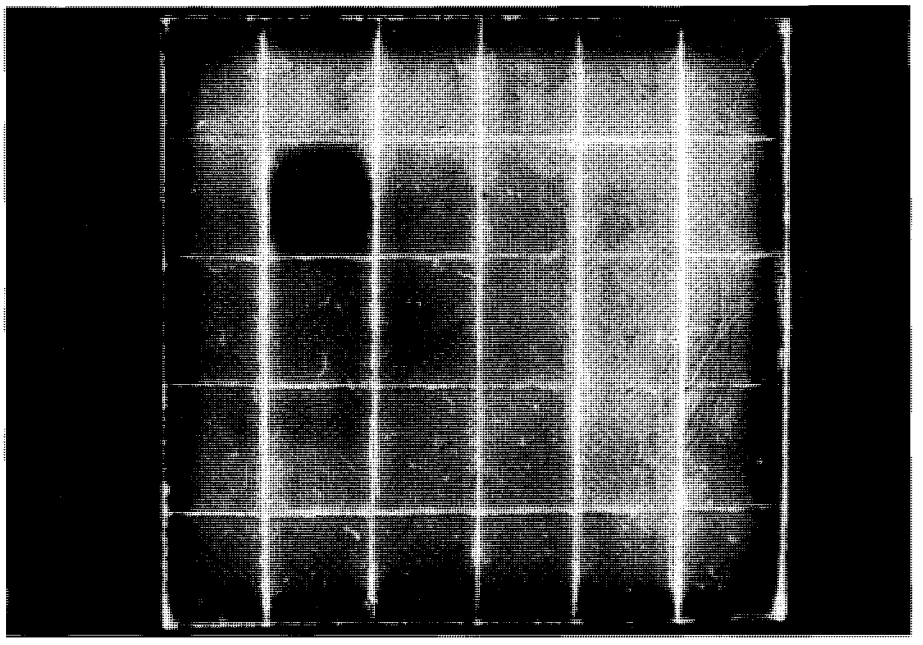

Fig. 10. The agar method for opacity-factor inhibition serotyping

5. Read and record the results. Inhibition is shown by a clear area on an opaque background.

Comments

- If the serum opacity reaction of the respective strain is too strong, dilute the culture supernatant or extract 1 : 4 with saline. Very high OF concentrations may require significantly higher dilutions.
- Weak OF-producers may often be satisfactorily tested by increasing the amount of supernatant or hydrochloric acid extract. If a threefold increase is not sufficient, more concentrated agar (e.g. 4%) must be used to achieve good solidification.

The agar method for OF-inhibition serotyping is illustrated in Fig. 10. In this test OF was incorporated into the agar and antisera were spotted on the agar surface. Of the 28 anti-OF sera used (including provisional types), only one inhibited the formation of opacity as evidenced by the area of clearing against the opaque background, indicating the OF type of the group A streptococcus.

8.4.2 Method 2

Equipment and supplies

— As described in section 8.3.

Reagents

— As described in section 8.3.

Method

1. Mix 1 ml of agar, 1 ml of pig or horse serum and 0.2 ml of anti-OF serum (the actual volume used will depend on the titre). These are the quantities

required for a slide measuring 5 × 5 cm. If a smaller slide or a Petri dish is used, the volumes should be adjusted accordingly.
2. Pour the mixture onto a slide or Petri dish and dry at 37 °C for 15 minutes.
3. Spot loopfuls of culture supernatant or hydrochloric acid extract of the OF+ strains under test onto the agar and incubate overnight at 37 °C in a moist chamber. Include a control slide without antiserum.
4. Read and record the results. An opaque area on a control slide that fails to develop on an antiserum slide indicates inhibition. Using this method, it is possible to test anti-OF sera for their specificity.

8.5 Tube method for opacity-factor detection and inhibition serotyping (104, 107, 112)

The presence of serum opacity factor and its inhibition is determined spectrophotometrically in test-tubes. This macromethod requires large quantities of ingredients and is not economical for routine use.

8.6 Microtitre plate method for opacity-factor detection and inhibition serotyping

The microtitre plate method has several advantages. It does not require time-consuming pre-preparation of agar slides or plates so that tests can be set up at very short notice. The microtitre plate can be used repeatedly until all wells have been utilized; even a single determination can therefore be run easily and economically. Furthermore, it is easy to control the strength of the OF reaction by varying the amount of OF added to the well. False-negative serotyping results can occur if too much OF is present (more than can be neutralized by the antibody) or if the OF reaction is too weak (making it difficult to detect inhibition) (*110*). The ability to quantify results spectrophotometrically can also be helpful. The main disadvantages of the method are the need for slightly larger volumes of antiserum, the costs of non-reusable microtitre plates compared with those of the reusable plates and slides used in the agar method, and the need for an ELISA microplate-reader if spectrophotometric quantification is desired.

Equipment and supplies

— Standard 96-well flat-bottomed microtitre plate
— Adjustable micropipette and tips, 0–20 µl
— Adjustable micropipette and tips, 20–200 µl
— Incubator at 35–37 °C
— Container (e.g. plastic bag, box with tight-fitting lid) into which the microtitre plate can be placed to maintain moisture during incubation.
— ELISA microplate-reader capable of producing a wavelength of 450 nm. If plates are to be read visually, a microtitre test reading mirror is useful.

Reagents

— Horse serum
— Culture supernatant, Lancefield hydrochloric acid extract or other opacity factor extract to be tested
— Source of anti-OF antibody (guinea-pig or rabbit hyperimmune serum or prescreened and tested human antisera)
— Normal serum for antibody control

— OF+ control strain (extract or supernatant of a known producer strain)
— Saline (sterile)

Method for OF detection

1. Add 100 µl of horse serum to the wells of the microtitre plate (The serum used must be prescreened and shown to give good opacity reactions with OF+ preparations. Adjusting the pH of the horse serum to 6.0 will enhance the opacity reaction and increase the sensitivity of the test, but with most strains this is not necessary.)
2. Add 10 µl of the extract or supernatant to be tested to the wells containing horse serum.
3. Add 10 µl of OF+ control extract to one well.
4. Leave the horse serum in one well without added extract or supernatant as a negative control.
5. Seal the plate and incubate overnight at 35 °C in a humid environment.
6. Remove the plate from the incubator and add 100 µl of saline to each well.
7. Read the results either visually (score reactions 0–4+) or with an ELISA spectrophotometric microplate-reader.

Use of the microtitre plate method for OF detection is illustrated in Fig. 11. A strong OF+ control was placed in well A1 and a horse serum control (without OF) in well H12. Negative reactions are shown by clear wells and positive reactions by wells with varying degrees of opacity.

Method for OF-inhibition serotyping

Results obtained using the microtitre plate method for OF-inhibition serotyping will be optimized if several controls are included. To accommodate these controls, each inhibition reaction being evaluated will require a total of four wells. These are: (1) a test well containing the OF extract being serotyped plus type-specific OF antiserum; (2) an antiserum control well

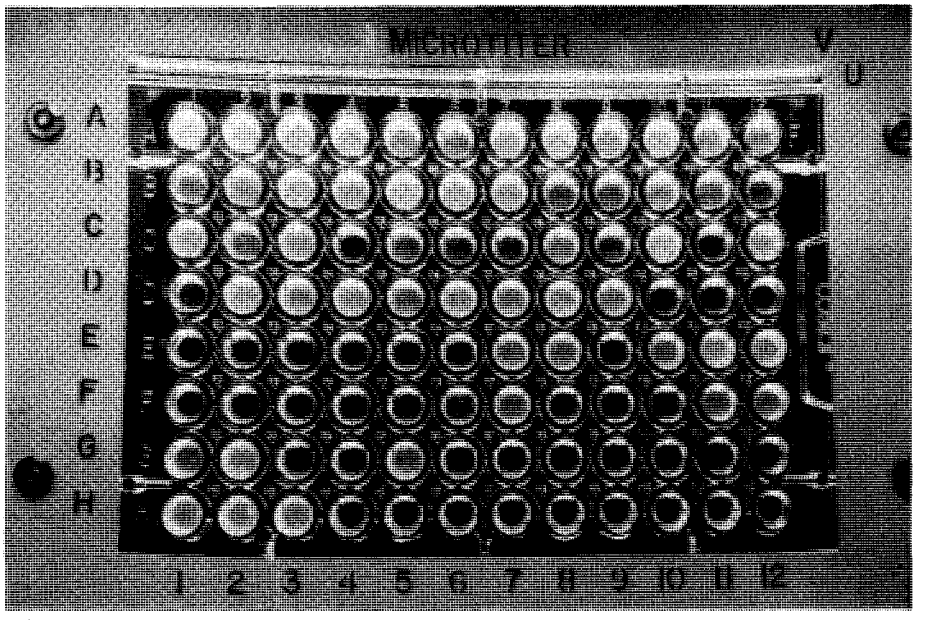

Fig. 11. The microtitre plate method for detection of serum opacity factor

containing type-specific antiserum plus saline; (3) an OF extract control containing the extract being serotyped plus a normal serum lacking OF antibody; and (4) a normal serum control containing normal serum lacking OF antibody, plus saline. All wells, of course, also contain horse serum.

Control 3 is necessary to show how much opacity the extract is capable of producing in the absence of type-specific antibody. Controls 2 and 4 are needed to evaluate any opacity added to the system by the type-specific or control sera. This is important because some human, rabbit and guinea-pig sera are sufficiently turbid (hyperlipaemic) that even the small amount added to the well causes opacity indistinguishable from a positive serum opacity reaction. In extreme cases this turbidity may mask true type-specific inhibition of the serum opacity reaction. If results are read using a spectrophotometer, the absorbance due to this nonspecific turbidity can be corrected for by treating control wells 2 and 4 as "blanks" relative to the corresponding wells 1 and 3. Once these serum control absorbance values have been subtracted, inhibition can be accurately evaluated by comparing the decrease in the corrected absorbance value (if any) from well 3 to well 1. Although this appears complicated, it is quite straightforward in principle. In practice, moreover, the absolute number of control wells required is not large: only one serum/saline control well (controls 2 and 4 above) is required on each plate for each corresponding serum, regardless of how many times that serum is used.

1. Add 10 µl of antiserum to wells.
2. Add 10 µl of normal serum to control wells.
3. Add opacity factor to wells. The amount added will depend on the strength of the opacity reaction observed during the detection test above. If the reaction was very strong (4+), add 2.5 µl of extract; if the reaction was 3+, add 5 µl, if 2+, add 10 µl, and if 1+, add 20 µl. This reduces the chances of very strong extracts "overwhelming" the antibody and also allows satisfactory testing of weak OF preparations. More accurate determination of optimal OF concentration can be obtained from absorbance readings. Very strong OF preparations may require significant dilution. This can be determined by preparing and testing sequential twofold dilutions of the OF, basing the final working dilution on the OF absorbance readings obtained.
4. Add 10 µl saline to saline control wells.
5. Seal the plate and incubate at 35–37 °C in a humid environment for 1 hour.
6. Add 100 µl of horse serum to each well.
7. Incubate overnight at 35–37 °C.
8. Add 100 µl of sterile saline to each well.
9. Read visually and/or in an ELISA microplate-reader at 450 nm.
10. If using an ELISA reader, calculate the inhibition as follows:
    (*a*) Subtract the absorbance of the control well containing type-specific antibody and saline (control 2) from the absorbance of all test wells containing that antiserum and OF extracts (control 1). This gives corrected test values for wells containing that antiserum.
    (*b*) Subtract the absorbance of the control well containing normal serum and saline (control 4) from the absorbance of all wells containing normal serum and OF extracts (control 3).
    (*c*) Positive inhibition of a specific extract by an antiserum is indicated when the absorbance value calculated in (*a*) is significantly less than the corrected value given by that extract in the presence of normal serum as calculated in (*b*). The reduction in absorbance required to verify inhibition will depend on the strength of the antiserum as well as the reproducibility of the test in the individual laboratory. An absolute minimum of 50% reduction in absorbance should be expected; normally, the reduction will be greater than 90%.

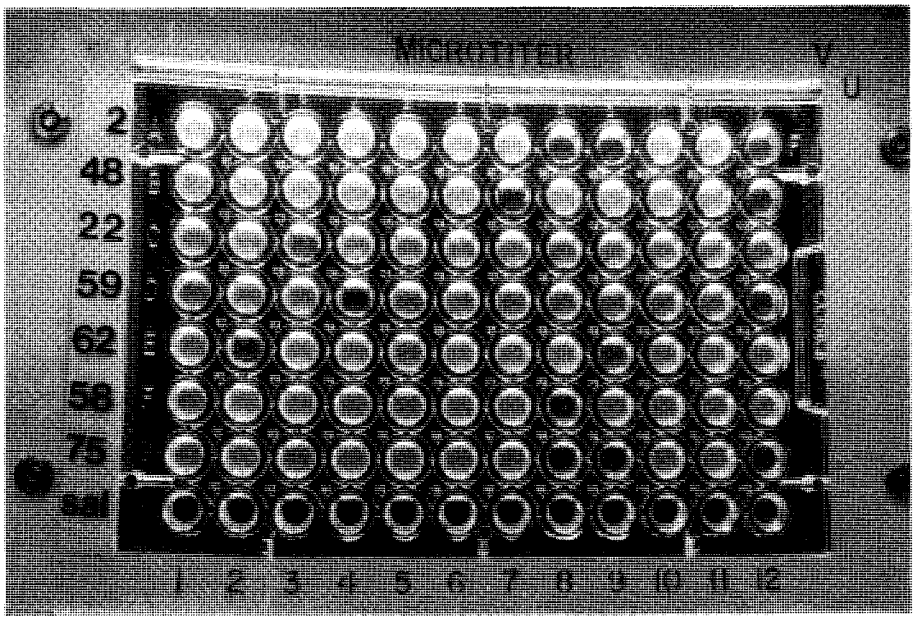

Fig. 12. The microtitre plate method for opacity-factor inhibition serotyping

(*d*) Normally, inhibition is readily discernible visually. Steps (*a*)–(*c*) above are of value when antisera are weak or turbid or when the opacity reaction itself is weak.

Use of the microtitre plate method for OF-inhibition serotyping is illustrated in Fig. 12. In this case, human sera were being screened for the presence of OF antibody to M types 2, 22, 48, 58, 59, 62 and 75. OF from each serotype was placed in different rows (A–G) and each of the human sera in a different column (2–12). Row H contains saline instead of OF, to measure any turbidity or opacity present in the sera being tested. Column 1 contains antibody-negative normal serum and is used to measure the amount of uninhibited opacity produced by each of the test strains. Presence of antibody in a serum is evidenced by inhibition of the opacity reaction resulting in a well with less opacity than is seen in the corresponding control well in column 1. For example, OF-2 was inhibited by antibody in three sera (wells A8, A9 and A12). Some sera were found to inhibit none of the strains tested (see column 6), whereas others inhibited several serotypes (see column 8).

9. Serological tests for group A streptococcal antibodies

9.1 *Immune response to streptococcal antigens*

By definition, true group A streptococcal infections are those that are followed by a specific immunological response — a significant increase in the titre of antibodies to at least one of the extracellular streptococcal antigens such as streptolysin O, deoxyribonuclease B, hyaluronidase or nicotinamide adenine dinucleotidase (*1*). These immune responses may be used to differentiate true infection from the carrier state. Acute rheumatic fever and post-streptococcal acute glomerulonephritis are virtually always associated with a rise in streptococcal antibody titres from acute to convalescent sera. For example, 80% of patients with rheumatic fever will demonstrate a rise in anti-streptolysin O titre. If the immune responses to three different antigens are measured, 97% of patients will show a rise in at least one. However, if antibiotic therapy was applied during the acute infection, the number of individuals demonstrating an immune response and the magnitude of the antibody responses may be much lower (*113*).

Antibody levels for each of the extracellular products are determined by neutralization tests. The rise in titre may begin soon after the onset of infection; maximum anti-streptolysin O titres usually occur 3–5 weeks after the onset of infection, but the anti-DNase B titre may not reach maximum until 6–8 weeks after onset. A gradual fall in titre usually begins within a few weeks, but the rate of decline varies considerably from patient to patient; anti-DNase B titres may remain elevated somewhat longer than anti-streptolysin O titres.

A rise in antibody titre can be interpreted as evidence of a recent group A streptococcal infection (for definition of a significant titre rise see sections 10–12 concerning individual antibodies). On the other hand, the absolute level of antibodies, whether high or low, may be misleading. The so-called upper limit for a "normal" titre is simply a statistical value for a given population at a given time and should be applied with caution. Thus, upper limits of normal may vary by age, season of the year and population studied. Normal individuals may have titres above the accepted "normal" values, representing past group A streptococcal infection(s). Antibody responses tend to be higher in patients with acute rheumatic fever than after uncomplicated streptococcal infections. Long-term monitoring of changes in antibody level after the peak has occurred, to determine whether a high titre persists or slowly or rapidly decreases, has very limited, if any, diagnostic or prognostic value.

The several weeks needed for the antibody response to develop renders serological evidence less helpful to the physician in the attempt to differentiate acute group A and non-group A streptococcal infection and to guide a therapeutic decision.

Failure to demonstrate a rise in titre makes the diagnosis of acute rheumatic fever or acute glomerulonephritis unlikely. However, in rheumatic fever there are recognized exceptions, for example attacks having as the sole manifestation either chorea or rheumatic carditis with insidious onset. These patients may first come to the attention of a physician only several months

after the group A streptococcal infection, when antibody titres could have decreased to lower levels. Given these exceptions, there should be great reluctance to make a diagnosis of acute rheumatic fever if adequate tests fail to produce serological evidence of recent streptococcal infection (2).

Most attacks of acute rheumatic fever and acute post-streptococcal glomerulonephritis appear between 1 week and 1 month after the beginning of the infection; the mean period of latency is usually 18 days for the former and 12 days for the latter following throat infection, but longer (2 or 3 weeks) for glomerulonephritis following skin infection. Thus, the chances of detecting a significant antibody response are best shortly (about 2–3 weeks) after the onset of the attack. It follows that repeated determinations of streptococcal antibodies on several serum samples obtained at weekly intervals are much more informative in detecting a trend of increase than examination of routine paired sera only. All available serum samples from the patient should also be examined simultaneously in the laboratory to minimize possible errors inherent in comparing results from several independent determinations. An antibody standard or reference serum with a known titre should be used as a control for each set of antibody determinations.

9.2 Handling and processing human sera for serological investigation

Human blood may without our knowledge contain dangerous pathogens such as hepatitis B virus or human immunodeficiency virus, and all samples of blood or serum must therefore be handled as potentially highly infectious material.

Venepuncture must be performed aseptically and the blood drawn into a closed system (e.g. a syringe). Non-haemolysed and bacterially uncontaminated serum samples are required for accurate serological investigation. Blood should be allowed to clot at room temperature and then held at 4 °C to allow retraction of the fibrin clot. On receipt in the laboratory, the serum sample should be checked for bacterial sterility by inoculation on a blood agar plate followed by incubation of the plate overnight at 37 °C and then overnight at room temperature. If bacterial contamination is present, the sample should be discarded. Only in special circumstances, such as in prospective studies with serial venepunctures when a new serum sample cannot be obtained, may a contaminated sample still be worth processing after elimination of the bacteria. This can be accomplished by filtration through a 0.22-μm membrane filter or, in the case of thermolabile organisms, by heating the sample to 56 °C for 30 minutes. However, interpretation of test results must take into account the possibility of a significant deterioration of the serum sample due to bacterial propagation. This is especially true with anti-streptolysin O, less so with anti-DNase B, anti-hyaluronidase or anti-M antibody tests. Some consider it advisable to heat all serum samples to 56 °C prophylactically immediately after separation from the fibrin clot as a means of preventing future growth of contaminating bacteria. This heating simultaneously inactivates the complement system, the activity of which may interfere in some antibody tests.

If multiple tests are planned, the sample should be distributed into at least two aliquots. The "working" sample may be kept at 4 °C with the addition of sodium azide (0.02% final concentration) to prevent contamination; the other aliquot(s) (for prolonged storage) should be kept frozen at ≤–20 °C without added preservative. Storage of the samples frozen without preservative

makes them suitable for use in the bactericidal test, if required. Before use, frozen aliquots should be thawed rapidly, preferably in a water-bath at 30–37 °C. However, repeated freezing and thawing eventually lowers the titre of antibodies present in the sample.

10. Determination of anti-streptolysin O

10.1 Introduction

The basis of anti-streptolysin O determination in serum is the neutralization of streptolysin O by anti-streptolysin O; the presence of residual unneutralized streptolysin O is demonstrated by the haemolysis of added erythrocytes. Only reduced (nonoxidized) streptolysin O is active in the reaction.

This determination is one of the best standardized serological methods and was the first to be developed for measuring antibodies to group A streptococci. An international standard of anti-streptolysin O is available from the Laboratory for Biological Standardization, Statens Seruminstitut, Copenhagen, Denmark; laboratory standards for testing can easily be derived by repeated testing against the international standard.

The range of "normal" values for anti-streptolysin O (as with other streptococcal antibodies) is variable and depends upon the age of the patient, geographical location, epidemiological setting and season of the year. Anti-streptolysin O titres are commonly reported in Todd units which represent the highest dilution of serum showing complete inhibition of haemolysis (*114, 115*). In the absence of specific information regarding the appropriate range of normal values calculated for each of these variables, single anti-streptolysin O titres are generally considered to be increased if they are at least 250 Todd units in adults and at least 333 Todd units in children over 5 years of age (2). It should be stressed that single lower titres do not exclude the possibility of streptococcal infection, since their comparison with a "normal value" may not be valid for the reasons given in section 9.1. It is therefore preferable to undertake acute and convalescent determinations. About 20% of infected individuals do not respond by an increase in anti-streptolysin O titre (*113*). Thus, a negative anti-streptolysin O titre alone cannot be used to rule out rheumatic fever or other streptococcal sequelae. Additional antibody tests (e.g. anti-DNase B, anti-hyaluronidase) may be required.

Streptococcal skin infections are usually followed by feeble anti-streptolysin O responses, probably because of an inhibitory action on streptolysin O by cholesterol and a number of related skin lipids (*116*). "Falsely" elevated anti-streptolysin O titres (not due to presence of antibody) may occur. Lipids present in serum (e.g. in patients suffering from hepatitis or nephrosis) act in the reaction as nonspecific inhibitors of streptolysin O. Some bacterial contaminants of a serum sample may have a similar effect on the titre by splitting serum lipoproteins and liberating cholesterol. Freezing and thawing of the sera may destabilize lipoproteins which can also alter the titre. Several methods can be used to remove the interfering lipids from serum samples, for example, dextran sulfate to absorb the active lipids (*117*), and simple chloroform lipid extraction (*118*). The latter appears to be easier to perform. Attention should be paid to the proper reduction of streptolysin O, as many of the falsely increased titres can be ascribed to its oxidation before or during the test. Elevated titres not representing group A streptococcal infections may also occur because this extracellular antigen is also produced by streptococci of groups C and G (*119*).

10.2 Spectrophotometric macromethod (120, 121)

The methods described in this section and in section 10.3 are those used in the Prague laboratory.

Principle

Dilute the serum to be examined in steps 1:2 and add one unit of previously titrated streptolysin O to each dilution. After the binding of streptolysin O to anti-streptolysin O, add standardized suspension of rabbit erythrocytes, incubate, measure the degree of haemolysis spectrophotometrically and calculate by interpolation the titre corresponding to 50% haemolysis. The table for the interpolation of values corresponding to 50% haemolysis is based on the assumption that the relationship of the logarithm of the dilution to the percentage of haemolysis is S-shaped. By means of the probit method, it is possible to calculate from these values the coefficient necessary for determining the serum dilution with 50% haemolysis.

Equipment and supplies

— Test-tubes
— Pipettes
— Water-baths at 37 °C and 56 °C
— Centrifuge
— Spectrophotometer
— Incubator at 37 °C
— Serum samples to be evaluated

Reagents

— Bovine serum albumin
— Distilled water
— Phosphate-buffered saline, pH 6.3–6.5, with albumin:

sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$)	7.2 g
disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)	9.02 g
sodium chloride	4.5 g
bovine serum albumin	1.0 g
distilled water	to 1000 ml

Before using a new batch of albumin, check to ensure that it does not influence the haemolytic activity of the streptolysin O.
— Streptolysin O
— Sodium dithionite ($Na_2S_2O_4$)
— Anti-streptolysin O standard
— Alsever solution:

sodium chloride	4.2 g
sodium citrate	8.0 g
citric acid	0.55 g
glucose	20.5 g
distilled water	to 1000 ml

Sterilize three times at 100 °C for 30 minutes. Store at 4 °C.
— Rabbit erythrocyte stock suspension. Withdraw rabbit blood by cardiac puncture, mix immediately with an equal volume of Alsever solution and store at 4 °C. This stock suspension is usable for at least 2–3 weeks. Before use, wash the erythrocytes three times in phosphate-buffered saline with albumin; centrifuge after each wash at approximately 150*g* for 10 minutes.

— Kalbak broth:
  (*a*) Mix 1000 g of ground beef heart with 2 litres of distilled water and keep at 4 °C overnight.
  (*b*) Boil for 10 minutes, filter through a layer of cotton wool and add 40 g of neopeptone per 1 litre of filtrate.
  (*c*) Adjust the pH to 7.3 and sterilize by filtration through a bacterial filter.
  (*d*) To 1 litre of filtrate add 40 ml of a solution prepared by dissolving 5 g of glucose, 5 g of sodium bicarbonate, 5 g of sodium chloride and 25 g of disodium hydrogen phosphate in 100 ml of distilled water and sterilizing by filtration through a bacterial filter.
— Sodium azide
— Streptococcal strain S84 or C203S

10.2.1 Streptolysin O preparation

Streptolysin O is available commercially. If needed, a potent and highly stable streptolysin O can be prepared in the laboratory as follows.

Method

1. Inoculate 1 litre of Kalbak broth with 5 ml of an 8-hour culture of *Streptococcus pyogenes* (group A) strain S84 or C203S.
2. Incubate at 37 °C overnight.
3. Check the supernatant for haemolysin as follows. Centrifuge 5 ml of the culture at approximately 1500 *g* for 30 minutes. To 1 ml of the supernatant add 1.4 mg of sodium dithionite, to reduce streptolysin O. To each of six test-tubes add 0.5 ml of phosphate buffered saline with albumin. Add 0.5 ml of reduced streptolysin O to the first test-tube, mix and transfer 0.5 ml into the next, and so on to the sixth test-tube (dilution 1:2, 1:4, etc., to 1:64). Add 1 ml of phosphate buffered saline with albumin and 0.5 ml of 5% suspension of rabbit erythrocytes in the same buffer to each test-tube, mix well and incubate in a water-bath at 37 °C for 60 minutes. If complete haemolysis is found in a dilution of 1:8 at least, the culture is suitable for further preparation.
4. Filter the culture through a bacterial filter.
5. To the filtrate add sodium azide to a concentration of 0.02% and store at 4 °C for at least 1 month before using for a titration (streptolysin S, if present, becomes inactive during this time). Streptolysin O retains a constant potency for many years when correctly stored at 4 °C.

Table 2. Example of results of spectrophotometric readings of haemolysis samples

Haemolysis (%)	Absorbance values, *A*, at 520 nm		
10	0.045	0.050	0.050
20	0.140	0.135	0.130
30	0.210	0.220	0.210
40	0.300	0.310	0.290
50	0.380	0.380	0.370
60	0.455	0.455	0.430
70	0.520	0.520	0.510
80	0.570	0.570	0.570
90	0.640	0.640	0.640
100	0.700	0.700	0.700

10.2.2 Calibration of the spectrophotometer

Each spectrophotometer must be individually calibrated. Tables 2–5 therefore provide only an example of such a procedure.

In the spectrophotometric method, the transmittance of samples with different percentages of haemolysis is determined and expressed as a function of the degree of haemolysis. It is most convenient, accurate and rapid to tabulate this relationship. For the purpose of calibration, however, the absorbance is measured rather than the transmittance (Table 2). From the values found, the linear regression is calculated using the method of least squares. Using this regression it is possible to convert every absorbance (and thus also transmittance) into a haemolysis value.

Table 3. Example of tabulated calculations for the regression coefficient formula

	x[a]	y[a]	xy	x^2
	10	45	450	100
	10	50	500	100
	10	50	500	100
	20	140	2800	400
	20	135	2700	400
	20	130	2600	400
	30	210	6300	900
	30	220	6600	900
	30	210	6300	900
	40	300	12000	1600
	40	310	12400	1600
	40	290	11600	1600
	50	380	19000	2500
	50	380	19000	2500
	50	370	18500	2500
	60	455	27300	3600
	60	455	27300	3600
	60	430	25800	3600
	70	520	36400	4900
	70	520	36400	4900
	70	510	35700	4900
	80	570	45600	6400
	80	570	45600	6400
	80	570	45600	6400
	90	640	57600	8100
	90	640	57600	8100
	90	640	57600	8100
	100	700	70000	10000
	100	700	70000	10000
	100	700	70000	10000
Total	1650	11840	829750	115500

[a] x, haemolysis values
y, values of measured absorbance at 520 nm (see Table 2) multiplied by 1000

Table 4. Example of the calculation of haemolysis values

T^a	A^a	A/b^a
100	0.000	
99	0.005	0.0007
98	0.010	0.0014
...		
...		
21	0.680	0.0942
20	0.700	0.0970

[a] *T*, transmittance; *A*, absorbance; *b*, regression coefficient

Table 5. Example of a conversion table from transmittance to haemolysis values (%)

Transmittance	0	1	2	3	4	5	6	7	8	9
20%	97	94	91	89	86	83	80	78	76	74
30%	72	71	69	67	65	63	61	59	58	56
...										
80%	13	12	12	11	10	10	9	8	8	7
90%	6	5	5	4	4	3	3	2	1	0

Procedure

Prepare a haemoglobin solution consisting of an approximately 8% suspension of washed rabbit erythrocytes in phosphate buffered saline with albumin (see above) and mix well. Take 1 ml and dilute 1 : 12 with distilled water. Read the transmittance (T) on the spectrophotometer at 520 nm. Adjust the T value to 20% by adding either erythrocyte mass or buffer to the erythrocyte suspension, and repeat the measurement. The solution of haemolysed erythrocytes with a T value of 20% represents the sample with 100% haemolysis. From the 100% haemolysis sample, prepare samples with 90%, 80%, 70%, etc. down to 10% haemolysis by dilution with distilled water. Measure the corresponding absorbance values (A) of the haemoglobin dilutions at 520 nm.

Repeat this procedure three times with blood from different rabbits, or at least one rabbit bled on different days, to obtain 30 measurements (Table 2). Considering the absorbance to be a function of haemolysis, calculate the regression coefficient (b) for this function using the following formula:

$$b = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{\left(\sum x\right)^2}{n}}$$

where x is the haemolysis percentage, y is the observed absorbance multiplied by 1000, and n is the number of measurements.

The following is an example of the calculation needed for the calibration of the spectrophotometer and for the construction of the table for converting transmittance values into haemolysis values.

Using the data from Table 2, calculate values for x, y, xy and x^2 as illustrated in Table 3. These values are then used to calculate the regression coefficient (b) as follows:

$$b = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} = \frac{829750 - \frac{1650 \times 11840}{30}}{115500 - \frac{1650^2}{30}} = 7.21414$$

The value A/b multiplied by 1000 gives the percentage of haemolysis. An example of a table showing conversion from transmittance to percentage haemolysis is provided by Table 5. It must be re-emphasized that the calculations shown in Tables 2–5 are examples only and that tables must be constructed in each laboratory and for each spectrophotometer.

10.2.3 Streptolysin O determination

Method

1. Prepare a standardized erythrocyte suspension as follows. Prepare an approximately 8% suspension of washed rabbit erythrocytes in phosphate buffered saline with albumin and standardize spectrophotometrically, adding either erythrocyte mass or buffer so that a sample diluted 1:12 with distilled water gives a transmittance of 20% sharp at 520nm (see above).
2. Add 0.5ml of streptolysin O to each of 10 auxiliary test-tubes.
3. Add phosphate buffered saline with albumin to each test-tube according to Table 6.
4. From each of these auxiliary dilutions transfer 0.5ml into a corresponding test-tube.
5. Rehydrate anti-streptolysin O standard with phosphate buffered saline with albumin to obtain one unit per ml. Immediately before used add sodium dithionite, 0.07%. Add 1ml of the resulting solution to each of the 10 test-tubes.
6. After mixing, incubate in a water-bath for exactly 15 minutes at 37°C.
7. Add 0.5ml of standardized suspension of erythrocytes, stir gently and incubate in a water-bath at 37°C for 1 hour. Stir once more during incubation.
8. After the incubation, centrifuge at 150g for 15 minutes to remove unhaemolysed erythrocytes.
9. Dilute 1ml of the supernatant from each test-tube with 2ml of distilled water.
10. Determine the transmittance and convert it into percentage haemolysis (see Table 5). The streptolysin O titre is indicated by the reciprocal

Table 6. Dilution scheme for streptolysin O (auxiliary test-tubes)

	Test-tube no.									
	1	2	3	4	5	6	7	8	9	10
Streptolysin O (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Buffer (ml)[a]	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50
Dilution	1:5.5	1:6.0	1:6.5	1:7.0	1:7.5	1:8.0	1:8.5	1:9.0	1:9.5	1:10.0

[a] Buffer, phosphate-buffered saline with albumin

value of the test-tube dilution for which the haemolysis value is closest to 50%.

10.2.4 Anti-streptolysin O determination

Method

1. Prepare a standardized erythrocyte suspension (see section 10.2.3).
2. Inactivate the serum samples at 56 °C for 30 minutes.
3. Dilute each serum 1:50 (50 µl in 2.5 ml of phosphate buffered saline with albumin) in an auxiliary test-tube.
4. Add 1 ml of phosphate buffered saline with albumin to each of five test-tubes.
5. Take 1 ml of the 1:50 diluted serum and add to the first test-tube.
6. Stir and transfer 1 ml into the next tube and so on to the fifth test-tube. This will give twofold serum dilutions 1:100, 1:200, 1:400, 1:800, 1:1600.
7. To each test-tube containing diluted serum add 0.5 ml of diluted freshly reduced streptolysin O (sodium dithionite, 0.14%) containing one unit in 0.5 ml.
8. Stir and incubate in a water-bath at 37 °C for exactly 15 minutes.
9. Add 0.5 ml of standardized erythrocyte suspension to each test-tube.
10. Stir gently and incubate in a water-bath at 37 °C for 1 hour. Stir once more during the incubation.
11. Identify by sight the test-tube with haemolysis just less than 50% and that with haemolysis just greater than 50%.
12. Centrifuge both test-tubes to remove unhaemolysed erythrocytes.
13. Remove 1 ml of the clear supernatant from each tube and dilute with 2 ml of distilled water.
14. Measure the transmittance of each solution with the spectrophotometer at 520 nm.
15. Using the previously constructed Table 5, convert the transmittance values into haemolysis values.
16. In the top row of Table 7 find the value closest to the percentage haemolysis of the tube with greater than 50% haemolysis. In the left-hand column, find the value closest to the percentage haemolysis of the tube with less than 50% haemolysis. At the intersection of the column and row representing these two values, find the coefficient to be used for determining the antistreptolysin O titre at 50% haemolysis. Multiply the reciprocal value of the dilution of the tube with less than 50% haemolysis by this coefficient to obtain the titre of the serum being tested.

Example of calculation: Haemolysis under 50% was found in the second test-tube (dilution 1:200) and over 50% in the third (dilution 1:400). After measurement it was found (Table 5) that haemolysis in the second test-tube was 32% and in the third 74%. The corresponding coefficient according to

Table 7. Coefficients for conversion of percentage haemolysis into anti-streptolysin O units

Haemolysis (%)	60	70	80	90	99.9
0.1	1.89	1.81	1.71	1.62	1.41
10	1.79	1.62	1.51	1.41	1.23
20	1.71	1.53	1.41	1.32	1.16
30	1.60	1.41	1.30	1.23	1.10
40	1.41	1.25	1.16	1.12	1.06

Table 7 was 1.41. Multiplying the reciprocal of the dilution of the second test-tube (200) by this coefficient (1.41) gives the value 282. The serum therefore contains 282 units of anti-streptolysin per ml.

The titration error of the spectrophotometric method is up to 30%. However, only rises in antibody titre of at least 100% correlated with untreated new acquisitions of group A streptococci (throat, nose) in children in prospective studies undertaken by the Prague laboratory.

10.3 Visual macromethod without use of spectrophotometer (122)

Equipment and supplies

— Test-tubes
— Pipettes
— Water-baths at 37 °C and 56 °C

Reagents

— Phosphate-buffered saline with albumin (see section 10.2)
— Streptolysin O
— Sodium dithionite ($Na_2S_2O_4$)
— Serum samples to be evaluated
— Anti-streptolysin O standard or control serum of known anti-streptolysin O titre
— Suspension of washed rabbit erythrocytes in phosphate-buffered saline with albumin, 5% (see section 10.2)

10.3.1 Streptolysin O determination

Method

1. Add 0.5 ml of streptolysin O to each of 10 auxiliary test-tubes.
2. Add phosphate-buffered saline with albumin according to the scheme given in Table 6.
3. Transfer 0.5 ml from each auxiliary test-tube to corresponding test-tubes.
4. Rehydrate the anti-streptolysin O standard with phosphate buffered saline with albumin to obtain 1 unit per ml. Immediately before use add sodium dithionite, 0.07%. Add 1 ml of the resulting solution to each of the 10 test-tubes.
5. After mixing, incubate in a water-bath at 37 °C for 15 minutes.
6. Add 0.5 ml of erythrocyte suspension.
7. After stirring, incubate in a water-bath for 1 hour at 37 °C. Stir once more during the incubation.
8. The highest streptolysin O dilution without haemolysis (i.e. the first test-tube with colourless supernatant after sedimentation of erythrocytes) is read as the end-point; the titre is the reciprocal value of this dilution.

10.3.2 Anti-streptolysin O determination

Method

1. Inactivate serum samples in a water-bath at 56 °C for 30 minutes.
2. Dilute 0.1 ml of serum sample with 0.9 ml of phosphate buffered saline with albumin (dilution 1 : 10).

3. Mix 0.5 ml of this dilution with 4.5 ml of phosphate buffered saline with albumin in an auxiliary test-tube (dilution 1:100).
4. Prepare 12 test-tubes.
5. Dilute the serum samples in geometric series in steps 1:1.25 in the following way. Transfer 1 ml of serum dilution 1:100 into the corresponding first test-tube. To the auxiliary test-tube add 1 ml of phosphate buffered saline with albumin (dilution 1:125) and mix well. Transfer 1 ml of this dilution into the second test-tube. To the auxiliary test-tube add 1 ml of phosphate-buffered saline with albumin (dilution 1:156) and mix well. Transfer 1 ml of this dilution to the third test-tube. Proceed in this way to the 12th test-tube. The resulting serum dilutions are 1:100, 1:125, 1:156, 1:195, 1:244, 1:305, 1:381, 1:476, 1:596, 1:744, 1:931 and 1:1163.
6. To each tube add 0.5 ml of diluted (immediately before use) reduced streptolysin O (sodium dithionite, 0.14%) containing 1 unit in 0.5 ml.
7. After mixing, incubate in a water-bath at 37 °C for 15 minutes.
8. Add 0.5 ml of erythrocyte suspension to each test-tube.
9. Stir and incubate in a water-bath for 1 hour at 37 °C. Stir once more during the incubation.
10. The highest serum dilution without haemolysis (the last test-tube with colourless supernatant after erythrocyte sedimentation) is read as the end-point. The anti-streptolysin O titre is the reciprocal value of this dilution.

10.4 Microtitre plate method (123)

The methods described in this section are those used in the Minneapolis laboratory.

Equipment and supplies

— Microdiluters, 25 µl, or appropriately sized multichannel or single channel micropipette and tips
— Droppers, 25 µl, or appropriately sized multichannel or single channel micropipette and tips
— Microtitre plates with U-bottomed wells
— Microtitre test reading mirror
— Centrifuge with microtitre plate carrier
— "Go-No-Go" test blotters (required only if microdiluters are used)
— Filter cartridges (required only if droppers are used)
— Incubator at 37 °C
— Water-bath at 56 °C
— Thermoplastic (stretchable plastic) film
— Refrigerator at 4 °C

Reagents

— Anti-streptolysin O buffer:

sodium chloride	7.40 g
potassium dihydrogen phosphate, anhydrous	3.17 g
disodium hydrogen phosphate, anhydrous	1.81 g
distilled water	to 1 litre
gelatin	1 g

Heat the buffer to near boiling to dissolve the gelatin. When cooled, adjust the pH to 6.5–6.7. Autoclave and store at 4 °C.
— Streptolysin O reagent. If using commercially prepared streptolysin O reagent, **do not prepare until immediately before use**, since the reagent is

oxygen-labile. Rehydrate following the manufacturer's instructions. Mix gently and use immediately, ideally within 15 minutes. The final concentration must be adjusted so that the anti-streptolysin O control serum gives a titre consistent with its predetermined value.

— Anti-streptolysin O standard. It is essential to use a human serum control with a known anti-streptolysin O titre. The titre of this control should be in the mid-range of the titres being run. It is also helpful to have available control sera of low and high titres for testing of sera with antibody levels outside the normal range.

— Defibrinated erythrocytes. Rabbit erythrocytes are commonly recommended although good success has also been achieved using sheep erythrocytes. Wash the cells twice with saline and then with anti-streptolysin O buffer. Draw off the supernatant and continue washing with buffer until the supernatant is clear. Mix by gently inverting the sealed tube. Centrifuge after each wash for 10 minutes at approximately 264 *g*. Remove the supernatant and resuspend the erythrocytes in buffer to give an approximately 5% suspension (0.75 ml of erythrocytes + 14.25 ml of buffer).

Method

1. Prepare working dilutions of human sera to be tested and of anti-streptolysin O control sera as follows.
   (*a*) Prepare 1:25 dilutions:
   1:25 0.1 ml of test or control sera and 2.4 ml of buffer
   (*b*) Inactivate the 1:25 dilution of the sera in a water-bath at 56 °C for 30 minutes.
   (*c*) From the 1:25 dilution prepare 1:30 and 1:40 dilutions of the test and control sera:
   1:30 0.2 ml of buffer and 1.0 ml of 1:25 dilution
   1:40 0.3 ml of buffer and 0.5 ml of 1:25 dilution
   Dilutions may be kept covered at 4 °C for up to one week.

Table 8. Example of the microtitre plate layout for anti-streptolysin O determination

	1	2	3	4	5	6	7	8	9	10	11	12
A	Patient serum 1			Patient serum 2			Patient serum 3			Patient serum 4		
	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160
B	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320
C	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640
D	1:800	1:960	1:1280	1:800	1:960	1:1280	1:800	1:960	1:1280	1:800	1:960	1:1280
E	Patient serum 5			Patient serum 6			Patient serum 7			Anti-streptolysin control		
	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160
F	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320
G	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640
H	1:800	1:960	1:1280	1:800	1:960	1:1280	1:800	1:960	1:1280	1:800	SO[a]	RBC[a]

[a] SO, streptolysin O control; RBC, red blood cell (erythrocyte) control

2. With a marker, divide the microtitre plate into eight sections of 3 × 4 wells (Table 8). Each section will be used to test one serum and the titre range in each section will be from 100 to 1280. On the lower right-hand side of the plate, mark off the last two wells (11H and 12H) for the streptolysin O and erythrocyte controls. These should be included in every plate. The anti-streptolysin control serum should be positioned in this section, in columns 10, 11 and 12 of rows E, F and G and column 10 of row H. The anti-streptolysin control should be included in at least every other plate. When preparing the sequential serum dilutions in this section of the plate, be careful not to add serum to the SO and RBC control wells. *Note*: If titres are suspected to be high or extremely variable, run three sera and a known high-titre control on each plate. Divide the plate into four sections of 3 × 8 wells, leaving the last two wells (11H and 12H) for streptolysin O and erythrocyte controls as before, and test a wider range of serum dilutions.
3. With the 25-µl dropper add 1 drop of the anti-streptolysin O buffer to each well except 12H, the erythrocyte control well, which should receive 2 drops. Alternatively, equivalent volumes can be added using a micropipette. Tap the plate five times on each side to distribute the drops in the wells. The plate can then be sealed with the thermoplastic film and stored at 4 °C until needed.
4. Using the microdiluters or multichannel pipette, add 25 µl of the first patient's serum dilutions (1:25, 1:30, 1:40) to the first three wells of the first row (columns 1, 2 and 3 of row A). The second patient's serum dilutions are added to columns 4, 5 and 6 of row A. Complete loading of plate using the layout in Table 8. Using the diluters or the multichannel pipette, serially dilute the sera by transferring 25-µl volumes row by row down the plate. When the serial dilutions are complete, check the diluters for bubbles and make a record if any are present. Drain each diluter on the "Go-No-Go" blotter, checking for accurate delivery. If a micropipette is used, tips must be changed for each serum sample and after each addition of buffer, streptolysin O and erythrocyte suspension. Remember to withdraw and discard 25 µl from the last row of each patient's dilution series to maintain equal volumes in all wells.
5. Add another drop of buffer (25 µl) to every well.
6. Immediately before use, rehydrate a vial of streptolysin O reagent with the appropriate amount of distilled water. Gently invert several times to mix. Add 1 drop (25 µl) of streptolysin O to all wells except the erythrocyte control. Tap gently on each side of the plate to mix.
7. Incubate for 30 minutes at 37 °C.
8. Add 25 µl of the erythrocyte suspension to every well. Tap each side of the plate gently to mix.
9. Incubate for 45 minutes at 37 °C.
10. Centrifuge the microtitre plates at approximately 225*g* for 3 minutes.
11. Read the plate using the test reading mirror and record results. The last well (highest dilution) without haemolysis is the titre. The streptolysin control should show complete haemolysis. The erythrocyte control should show no haemolysis.

Comments

Most human sera will have anti-streptolysin O titres of between 100 and 1280 Todd units and can be successfully analysed using the above dilution scheme. When it is necessary to determine accurately the titre of an antiserum whose values fall outside this range, the dilution schemes shown in Table 9 can be used. If the sera being tested vary widely in titre, it may be necessary to test fewer sera per plate and cover a wider dilution range. In such situations the

Table 9. Suggested dilution schemes for sera of low and high titre

Titre	Dilution		
Low	1:12.5	1:15	1:20
	1:25	1:30	1:40
	1:50	1:60	1:80
	1:100	1:120	1:160
High	1:400	1:480	1:640
	1:800	1:960	1:1280
	1:1600	1:1920	1:2560
	1:3200	1:3840	1:5120

Table 10. Conversion of antibody titres from Todd units to $\log_{10}$

Todd units	$\log_{10}$	Todd units	$\log_{10}$	Todd units	$\log_{10}$
12.5	1.10	100	2.00	800	2.90
15	1.18	120	2.08	960	2.98
20	1.30	160	2.20	1280	3.11
25	1.40	200	2.30	1600	3.20
30	1.48	240	2.38	1920	3.28
40	1.60	320	2.51	2560	3.41
50	1.70	400	2.60	3200	3.51
60	1.78	480	2.68	3840	3.58
80	1.90	640	2.81	5120	3.71

plate may be divided into four sections of three columns each, with each section covering a dilution range of 1:50 to 1:10240.

The relationship between initial serum dilution and final dilution in the anti-streptolysin O test plate may not be immediately apparent. The following example may help to explain this. A 25-µl volume of the initial 1:25 serum dilution is added to a well containing 25 µl of anti-streptolysin O buffer. This results in a serum dilution of 1:50 in a well containing 50 µl. A 25-µl volume of this dilution is then removed to prepare additional serum dilutions. An additional 25 µl of anti-streptolysin O buffer is then added to the well, bringing the serum dilution to 1:100. It is at this point that the enzyme is added. Thus, all initial serum dilutions prepared in steps 1(*a*) and 1(*c*) above yield dilutions in the plate of four times their initial value (i.e. 1:25 becomes 1:100, etc.).

Anti-streptolysin O titres are often reported in $\log_{10}$ units rather than in Todd units. Conversion is accomplished by simply calculating the logarithm to the base 10 of the Todd unit titre. Commonly encountered titres and their corresponding log values are shown in Table 10.

11. Determination of anti-deoxyribonuclease B

11.1 Introduction

Group A streptococci produce four antigenically different deoxyribonucleases (DNases) designated A, B, C and D. The most important is DNase B, the largest amounts of which are produced by group A strains associated with human infections. The commonly used micromethod for anti-DNase B determination is based on the formation of a coloured complex between deoxyribonucleic acid (DNA) substrate and methyl green. Digestion of the substrate by DNase B is accompanied by loss of colour. If antibodies are present in the serum they prevent the enzyme from attacking the substrate and the coloured complex is unchanged (*124, 125*). Commercial kits for the determination of anti-DNase B titres are available.

Anti-DNase B responses to group A streptococcal infections of the upper respiratory tract occur as frequently as anti-streptolysin O responses but, in contrast to the latter, are also commonly observed following group A streptococcal skin infections. Since no international reference anti-DNase B serum is currently available, a direct comparison of titre values is possible only between laboratories using the same standard or reference sera.

The "upper limit of normal" for anti-DNase B titre — as with other group A streptococcal extracellular antibodies — depends on a number of factors, including the epidemiological circumstances, and demographic and geographical variables. DNase B titres of 240 or greater in school-age patients and 120 units or greater in adult patients are viewed as elevated in many parts of the USA (*126*). The limits differ in other settings, however; for example, in one stratified serological survey of a population in Czechoslovakia in 1973, the upper limits of normal were as high as 400 units in children over 5 years and 200 units in adults. Although production of anti-DNase B is not strictly limited to group A streptococci, most increases in anti-DNase B titres occur as responses to group A streptococcal infections (*115*).

11.2 Microtitre plate method 1

The methods described in this section are those used in the Prague laboratory.

11.2.1 Determination of DNase B concentration

Equipment and supplies

— Test-tubes
— Pipette droppers, 25 µl and 50 µl
— Microtitre plates with U-bottomed wells
— Micropipettes, 40–200 µl
— Pipettes, 2 ml
— Funnel
— Distilled water
— Freezers at –20 °C and –70 °C
— Bacterial filter
— Microdiluters, 25 µl

— Dialysis tubes
— Incubator
— Test reading mirror

Reagents

— Nessler reagent
— DE 52 cellulose
— Chloroform
— Anti-DNase B standard
— Buffer (1): tris–hydrochloric acid buffer, 0.1 mol/l, pH 7.8 with Mg^{2+} ions, 0.02 mol/l:

tris	1.21 g
magnesium chloride	0.4 g
distilled water	to 100 ml

Adjust the pH to 7.8 with hydrochloric acid, 4 mol/l.
— Buffer (2): tris–hydrochloric acid buffer, 0.01 mol/l, pH 7.8 with Ca^{2+} ions, 0.001 mol/l, Mg^{2+} ions, 0.001 mol/l, and 0.1% bovine serum albumin:

tris	1.21 g
calcium chloride	0.22 g
magnesium chloride	0.2 g
bovine serum albumin	1 g
distilled water	to 1000 ml

Adjust the pH to 7.8 with hydrochloric acid, 4 mol/l, and add 0.2 g of sodium azide.
— Buffer (3): acetate buffer, 0.02 mol/l, pH 4.2:

sodium acetate	136 mg
distilled water	to 50 ml

Adjust the pH to 4.2 with hydrochloric acid, 1 mol/l.
— Methyl green dye:
(*a*) Dissolve 100 mg of methyl green in 10 ml of buffer (3).
(*b*) Extract repeatedly with chloroform using a separatory funnel; when the chloroform phase is colourless, the separated dye–buffer phase is ready for use; store at 4 °C.
— DNA–methyl green substrate (0.1% solution of DNA with 0.01% methyl green):
(*a*) Take 100 mg of DNA (highly polymerized, sodium salt, from calf thymus) cut into small pieces.
(*b*) Allow to swell in 50 ml of distilled water with 0.02% sodium azide at room temperature overnight; swirl occasionally.
(*c*) Add 50 ml of buffer (1) and 1 ml of methyl green solution with stirring; stand at room temperature and allow the complex to form.
(*d*) Distribute a small amount into test-tubes and store at –20 °C.
— Streptococcal DNase B enzyme:
(*a*) Inoculate 1 litre of Todd–Hewitt broth with 5 ml of an 8-hour culture of *Streptococcus pyogenes* (group A) strain C 203S and incubate at 37 °C overnight. Filter the culture through a bacterial filter.
(*b*) To the filtrate add ammonium sulfate to 57% saturation (430 g per 1000 ml) with stirring, and allow the precipitate to form at 4 °C overnight.
(*c*) Separate the precipitate by centrifuging at approximately 1500 *g* for 15 minutes, dissolve it in distilled water (in approximately 5% of the original volume) and dialyse against distilled water to remove ammonium ions (e.g. test with Nessler reagent).
(*d*) Assay crude enzyme activity: Add 25 µl of buffer (2) to 10 wells on a microtitre plate; using a microdiluter transfer 25 µl of enzyme diluted 1:100 into the first well, rotate the diluter several times and transfer

25 µl to the next well and so on to the tenth well. To each well add 25 µl of buffer (2) and 50 µl of DNA–methyl green substrate. Mix by tapping each side of the plate and incubate in a wet chamber at 37 °C overnight. The reciprocal value of the highest enzyme dilution with 50% substrate digestion (see below) is the crude enzyme titre.

(*e*) Purify crude enzyme (with a titre over 12000) chromatographically at 4 °C on DE 52 cellulose using the batch adsorption method (127). Separate the solution containing DNase B from cellulose, distribute in aliquots measured precisely (e.g. 0.3 ml) into test-tubes for lyophilization and keep at –70 °C until freeze-dried.

— Sodium azide

Method

1. Rehydrate the lyophilized DNase B enzyme to the original volume with distilled water.
2. Determine the approximate titre of the purified enzyme in an assay using large steps between dilution in arithmetic series, e.g. 1:50, 1:100, 1:150, 1:200, 1:250, 1:300.
3. Using a pipette dropper, transfer 25 µl of each enzyme dilution into a corresponding well of a microtitre plate.
4. To each well add 25 µl of a standard of anti-DNase B diluted with buffer (2) so as to obtain 1 unit in 1 ml.
5. After mixing, incubate in a wet chamber at 37 °C for 15 minutes.
6. Add 50 µl of substrate to each well, mix by tapping each side of the microtitre plate and incubate in a wet chamber at 37 °C overnight.
7. The approximate enzyme titre is the dilution that would lie between the last colourless and the first coloured content of the wells.
8. Repeat the whole titration procedure with a series of enzyme dilutions using smaller steps between dilutions (e.g. 1:50, 1:75, 1:100)
9. The colour intensity is read as follows: 3+, no change in colour (no digestion of substrate); 2+, moderate decoloration (approximately 50% digestion); 1+, some colour left (over 50% digestion); 0, colourless well contents (complete digestion of substrate).
10. The titre is determined by the reciprocal value of the highest enzyme dilution that, in the presence of 1 unit of antibody, causes approximately 50% digestion of the substrate (colouring 2+).

11.2.2 Determination of anti-DNase B titre in patient sera

Equipment and supplies

— Test-tubes
— Pipette droppers, 25 µl and 50 µl
— Microdiluters, 25 µl
— Microtitre plates with U-bottomed wells
— Microtitre test reading mirror
— Wet chamber at 37 °C
— Incubator at 37 °C

Reagents (see section 11.2.1)

— Buffer (2)
— Serum samples previously heat inactivated at 56 °C for 30 minutes
— DNase B enzyme
— DNA–methyl green substrate
— Control serum of known anti-DNase B titre

Method

1. Dilute each serum specimen in a test-tube 1:25 (50 µl of serum and 1.2 ml of buffer (2)).
2. From this dilution prepare dilutions of 1:50 and 1:75 in buffer (2). Titrate the sera in the horizontal rows of the microtitre plate.
3. Add 25 µl of buffer (2) to all wells of the plate using a pipette dropper.
4. Use a 25-µl microdiluter to transfer an equal volume of serum diluted 1:50 into the first well (be careful to dip only the diluter tip) and, after mixing, transfer 25 µl into the third well.
5. Continue this procedure along the row in odd-numbered wells.
6. Similarly, prepare serum dilutions from the starting dilution of 1:75 in even-numbered wells. In this way the following serum dilutions are prepared: 1:100, 1:150, 1:200, 1:300, 1:400, 1:600, 1:800, 1:1200, 1:1600, 1:2400.
7. Using a 25-µl pipette dropper, add to each well DNase B enzyme diluted immediately before use so as to contain 1 unit per ml.
8. Mix by tapping each side of the plate and incubate in a wet chamber at 37 °C for 15 minutes.
9. Add 50 µl of DNA–methyl green substrate to each well, mix and incubate in a wet chamber at 37 °C overnight.
10. Include the following controls:
    (*a*) enzyme control: 25 µl of buffer (2), 25 µl of diluted enzyme and 50 µl of substrate solution (must be colourless after incubation)
    (*b*) substrate control: 50 µl of buffer (2) and 50 µl of substrate solution (must retain green colour after incubation)
    (*c*) control serum of known anti-DNase B titre (e.g. 400 units).
11. Read using the test reading mirror.
12. The titre corresponds to the reciprocal value of the serum dilution that causes an approximately 50% inhibition of the enzyme (colour 2+). If there is a steep colour transition (colour 3+ followed by total loss of colour), read the last well with total enzyme inhibition (3+) as the end-point.
13. An increase in anti-DNase B titre of at least 100% if determined in the same test, or at least 200% if determined in independent runs, is considered to be significant.

11.3 Microtitre plate method 2

The methods described in this section are those used in the Minneapolis laboratory.

Equipment and supplies

— Microdiluters, 25 µl, or multichannel or single-channel micropipette and tips
— Pipette droppers, 25 µl, or multichannel or single-channel micropipette and tips
— Pipette droppers, 50 µl, or multichannel or single-channel micropipette and tips
— Microtitre plates with U-bottomed wells
— Microtitre test reading mirror
— "Go-No-Go" test blotters (not required if micropipettes are used)
— Pipette filter cartridges (not required if micropipettes are used)
— Thermoplastic (stretchable plastic) film
— Adhesive plate-sealing film
— Water-bath at 63 °C
— Incubator at 37 °C

Reagents

— Tris buffer, pH 7.6:

tris	2.4228 g (0.01 mol/l)
magnesium sulfate, anhydrous	0.7203 g (0.003 mol/l)
calcium chloride	0.6660 g (0.003 mol/l)
distilled water	2000 ml

Adjust the pH to approximately 7.4. Add 2 g of gelatin (0.1%) and allow it to soften for 10 minutes. Heat until the gelatin is completely dissolved. Divide the buffer into two 2-litre flasks and autoclave at 121 °C for 18 minutes. Cool to room temperature and adjust the pH to 7.6. Store at 4 °C. Pour off the volume of buffer needed for the day into a 250-ml beaker. Keep the buffer on ice and check and adjust as necessary to maintain a pH of 7.6. The buffer will be used for all serum and enzyme dilutions.

— Imidazole buffer:

imidazole	13.616 g (0.1 mol/l)
distilled water	2000 ml

Adjust the pH to 7.7 and store at 4 °C.

— Methyl green dye. Dissolve 544.32 mg of sodium acetate trihydrate in 200 ml of distilled water. The pH will be about 7.6; adjust to 4.2 with hydrochloric acid, 10 mol/l. Add 500 mg of methyl green to 100 ml of this acetate buffer.

Note: The methyl green used has the following specifications: C.I. 42590; total dye content 84%; if the dye content varies from this value, the amount of methyl green may have to be adjusted. Extract repeatedly with chloroform using a separatory funnel. Do not lose the dye–buffer phase. When colour no longer appears in the chloroform, the dye solution is ready for use.

— DNA ("completed"):

(*a*) Use highly purified DNA from calf thymus. Weigh 150 mg of DNA, handling it with two sets of very clean forceps (the amount of DNA added has ranged from 100 to 200 mg depending on quality). Place the weighed DNA into a sterile 250-ml flask and add 50 ml of distilled water.

(*b*) Place 120 mg of anhydrous magnesium sulfate in a 125-ml flask. Add 50 ml of imidazole buffer and swirl to dissolve. This solution will be cloudy at first and will clear within about 15 minutes.

(*c*) Place the flasks from (*a*) and (*b*) at 4 °C overnight or over the weekend (but no longer) to soak and thereby soften the DNA. Place the flask from (*a*) on a magnetic stirrer in the cold room and allow to stir gently for 4–6 hours. If the DNA is not completely dissolved, remove from the stirrer and again allow the DNA to stand overnight. Stir again the next morning for 1 hour. Swirl the flask from (*b*) and add to the DNA. Stir for 5 minutes. This is the "completed" DNA.

Note: The completed DNA must be tested before use in the DNase B test using the procedure described in section 11.3.2.

— DNA–methyl green substrate. Swirl the methyl green solution to mix and add 2 ml per 100 ml of completed DNA. The actual volume added may need to be adjusted to achieve the proper colour intensity (a colour that yields a clear end-point). Stir for 5 minutes. Store at 4 °C. Pour off enough DNA–methyl green substrate for the week into a small flask or bottle. Gently remove the required amount using a dropper or pipette. Take care to maintain the preparation free from contamination.

— Daily DNase B enzyme dilution (section 11.3.1)

— Control sera of known anti-DNase B titre (it is preferable to use two, one with a high titre and one with a low titre)

— Sera to be tested

11.3.1 Determination of DNase B enzyme concentration

Method

1. Streptococcal DNase B is isolated by batch adsorption (*127*). The enzyme may be lyophilized and stored at 4 °C or stored in liquid form at ≤–20 °C; it remains stable for at least 6 months.
2. Prepare a 1 : 100 dilution of the DNase B enzyme in tris buffer. Determine the final working dilution of DNase B as follows in steps 3–9.
3. Take a microtitre plate and add 25 µl of tris buffer to each well in column 1, rows A to H.
4. To the first well, 1A, add 25 µl of the 1 : 100 enzyme dilution. Serially dilute from 1A to 1H using the 25-µl microdiluter or micropipette.
5. Add 25 µl of tris buffer to each well.
6. Add 50 µl of the DNA–methyl green substrate to each well. Tap, seal with a plate sealer, and incubate at 37 °C. After 5 minutes remove the plate and tap again to ensure thorough mixing of well contents.
7. After 20 hours of incubation at 37 °C, do a preliminary reading on the plate. Then store the plate at room temperature for an additional 24 hours before the final reading, which is used to calculate the final enzyme dilution.
8. The lowest dilution well that shows definite colour (3+) is the end-point of the enzyme reaction. This dilution would be 200 if it occurred in well 1A, 400 in well 1B, 800 in well 1C, 1600 in well 1D, 3200 in well 1E, 6400 in well 1F, 12800 in well 1G and 25600 in well 1H. A fourfold concentration of this end-point dilution should approximate the working dilution, which is calculated as follows. If the end-point (3+ to 4+ colour) is in well 1F, this would correspond to an enzyme dilution of 1 : 6400. Therefore, the approximate daily working enzyme dilution would be 1 : 1600. This fourfold concentration allows for sufficient enzyme to degrade the DNA but not such excessive amounts that normal antibody levels are unable to neutralize the reaction.
9. Human control sera with known anti-DNase B titres, preferably of both high and low titre, are used to determine the exact working dilution of enzyme. This is done by testing several closely spaced enzyme dilutions around the range of the previously determined approximate daily working dilution. For example, if step 8 above gave an approximate working dilution of 1 : 1600, the range tested might be 1 : 1200 to 1 : 2000. The final dilution that gives titres for the standard human control sera equal to their established values is used as the exact daily working enzyme dilution.
10. The final daily working dilution is determined by its ability to deliver the previously established titres of the control sera. The consistent and reproducible known titre of the human control serum is the constant. Variables necessitating a change in DNase B concentration may include a new batch or source of DNase B or a new lot of DNA. Adjustments are made to attain the required titres in tests using the standard human control sera.

11.3.2 Testing of DNA

Each new lot of DNA must be tested for clot formation before use in the microtitre plate test. (The DNA may be tested either before or after addition of the methyl green. It should be freshly made and free of chloroform.)

Method

1. In a 16 × 100 mm test-tube combine 0.4 ml of tris buffer with 0.4 ml of completed DNA and mix well.

2. Add 1.0 ml of cold absolute or 95% ethanol and mix vigorously.
3. If a large firm clot appears within a clear filtrate, the DNA is probably suitable for use in the DNase B test.

11.3.3 Determination of the anti-DNase B titre in human sera

Method

1. Adjust the pH of the tris buffer to 7.6.
2. Prepare 1:25 dilutions of sera to be tested using 0.1 ml of serum and 2.4 ml of tris buffer.
3. Heat-inactivate the 1:25 dilutions for 30 minutes at 63 °C.
4. From the heat-inactivated 1:25 dilutions, prepare 1:30 and 1:40 dilutions:
   1:30 0.2 ml of buffer and 1.0 ml of the 1:25 dilution
   1:40 0.3 ml of buffer and 0.5 ml of the 1:25 dilution
   Dilutions may be kept covered at 4 °C for up to 1 week.
5. With a marker, divide the microtitre plate into eight 3 × 4 well sections. This will allow either seven test sera and one control serum or six test sera and two control sera as well as enzyme and substrate control wells to be included on each plate (Table 11). If the sera being tested cover a wide range of titres, it may be necessary to test fewer sera per plate and cover a wider range of dilutions.
6. Using a dropper or micropipette, add 25 µl of tris buffer to each well of the microtitre plate except the DNA substrate control well, which should receive 50 µl.
7. Tap each side of the plate gently to distribute the drops evenly in the wells.
8. If multiple plates are being run or any delay is encountered, cover the plate tightly with thermoplastic film and keep cold until use. Plates may be held for up to 3 hours if kept cold and tightly sealed.
9. Using the microdiluters or multichannel pipette add 25 µl of the first patient's serum dilutions (1:25, 1:30 and 1:40) to the first three wells of

Table 11. Example of the microtitre plate layout for anti-DNase B determination

	1	2	3	4	5	6	7	8	9	10	11	12
A	Patient serum 1			Patient serum 2			Patient serum 3			Patient serum 4		
	1:50	1:60	1:80	1:50	1:60	1:80	1:50	1:60	1:80	1:50	1:60	1:80
B	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160
C	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320
D	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640
E	Patient serum 5			Patient serum 6			Human serum control			Human serum control		
	1:50	1:60	1:80	1:50	1:60	1:80	1:50	1:60	1:80	1:50	1:60	1:80
F	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160	1:100	1:120	1:160
G	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320	1:200	1:240	1:320
H	1:400	1:480	1:640	1:400	1:480	1:640	1:400	1:480	1:640	1:400	Enzyme control	DNA control

the first row (columns 1, 2 and 3 of row A). The second patient's serum dilutions are added to columns 4, 5 and 6 of row A. Using the diluters or multichannel pipette, serially dilute the sera by transferring 25-µl volumes row by row down the plate. When the serial dilutions are complete, check the diluters for bubbles and record if present. Drain each diluter on the Go-No-Go blotter, checking for accurate delivery. If a micropipette is used, tips must be changed for each patient and after each addition of buffer and enzyme, and substrate. Remember to withdraw and discard 25 µl from the last row of each patient's dilution series to maintain equal volumes in all wells.

10. Add 25 µl of the DNase B daily dilution (section 11.3.1) to all wells except the DNA–methyl green substrate control well.
11. Mix by gently tapping each side of the plate and then seal with stretchable plastic film.
12. Incubate at 37 °C for a total of 15 minutes, but tap the plate again after 7 minutes to ensure thorough mixing.
13. Add 50 µl of DNA–methyl green substrate to all wells. Gently tap the sides of the plate to mix.
14. Seal the plate securely with adhesive plate sealer, tap the plate again to mix the reagents in the wells, and place in an incubator at 37 °C. After 5 minutes mix thoroughly once more by tapping the sides of plate. Incubate for 20 hours at 37 °C. Incubation time may range from 19 to 24 hours but should be consistent for each test.
15. Read and record the results. The colour gradation ranges from 0 (no colour) to 4+ (strongest colour). The enzyme control should be 0 and the substrate control 4+. The last well (highest dilution) with a reading of 4+ or 3+ is the end-point and titre. This reading is considered to be a preliminary test result only.
16. Reincubate plate for an additional 18–24 hours at room temperature. Read and record the results as in step 15 above. This reading is reported as the final test result.

Comments

Most human sera will have an anti-DNase B titre of between 50 and 640 and can be successfully analysed using the plate layout shown in Table 11. If it is necessary to determine accurately the titre of an antiserum whose values fall outside this range, the dilution scheme shown in Table 9 can be used. If the sera being tested cover a wide range of titres, it may be necessary to test fewer sera per plate and cover a wider range of dilutions. For example, one serum can be tested in a set of wells covering three columns and 12 rows (36 wells). The range of dilutions covered would be from 1 : 25 to 1 : 5120. The relationship between the patient's initial serum dilution and the corresponding microplate well titre can be explained as follows. A 25-µl volume of a 1:25 serum dilution is added to a well that contains 25 µl of tris buffer. This gives an effective serum dilution of 1:50 in the well to which DNase B enzyme is added. Thus, all initial dilutions prepared in section 11.3.3, steps 2 and 4, yield dilutions in the plate of twice their initial value (i.e. 1:25 becomes 1:50, etc.).

Anti-DNase B titres are often reported in $\log_{10}$ units rather than in the standard reciprocal dilution units shown above. The conversion from standard units to log units is as shown in Table 10.

12. Determination of streptococcal anti-hyaluronidase

12.1 Introduction

Streptococcal anti-hyaluronidase is the antibody to streptococcal hyaluronidase, an enzyme that splits hyaluronic acid or its salt. Determination of streptococcal anti-hyaluronidase titre is based on the neutralization of hyaluronidase activity by the antibody in the human test serum (*128*). Hyaluronidase must be titrated before use in the test. The turbidimetric method is commonly used. It is based on the turbidity formed by hyaluronic acid or its salt with acidified protein solution in an appropriate buffer. The neutralization of the enzyme by specific antibody is demonstrated by a decrease in turbidity. A commercial test is also available.

Although only strains of serotypes 4 and 22 produce appreciable amounts of hyaluronidase *in vitro*, most strains of group A streptococci have the ability to produce this enzyme *in vivo*. It is antigenically distinct from hyaluronidases of other serological groups of streptococci, and its neutralization by antibody is therefore considered specific for group A (*129, 130*). The anti-hyaluronidase responses occur with a frequency similar to that of anti-DNase B responses and follow both throat and skin infections. Only a titre increase of at least 400% is accepted as significant. Less is known about the titre ranges in the general population, although 400 units can be considered the upper limit of normal titre in children of school age. Normally occurring nonspecific inhibitor of the hyaluronidase in human sera is inactivated by heating at 56 °C for 30 minutes. Nonspecific streptococcal anti-hyaluronidase titres induced by bacterial contamination of the serum sample are rarely suspected.

There is no international standard for streptococcal anti-hyaluronidase.

12.2 Hyaluronidase titration

Equipment and supplies

— Test-tubes
— Incubator or water-bath at 37 °C
— Water-baths at 60 °C and 100 °C
— Spectrophotometer
— Pipettes
— Gas or electric heaters
— Autoclave
— Bacterial filter
— Centrifuge
— Dialysis bags
— Freezer at –30 °C

Reagents

— Buffer (1): acetate buffer, 0.1 mol/l, pH 6.0, with sodium chloride, 0.15 mol/l:

acetic acid, glacial	6.0 g
sodium hydroxide, 1 mol/l	100 ml

sodium chloride	8.8 g
distilled water	to 1000 ml

— Buffer (2): acetate buffer, 0.5 mol/l, at pH 4.2:

acetic acid, glacial	30 g
sodium hydroxide, 0.1 mol/l	140 ml
distilled water	to 1000 ml

— Hartley broth:
  (*a*) Mix 1000 g of minced beef heart with 1500 ml of distilled water and keep overnight at 4 °C.
  (*b*) Heat the mixture at 80 °C for 90 minutes.
  (*c*) Add to the hot mixture 15.4 g of sodium carbonate dissolved in 1500 ml of distilled water. Cool to 45 °C.
  (*d*) Add 40 mg of trypsin dissolved in 40 ml of phosphate buffered saline at pH 7.3, and 34 ml of chemically pure chloroform and keep at 37 °C for 6 hours (digestion), mixing from time to time.
  (*e*) Keep overnight at 4 °C.
  (*f*) Neutralize with 40 ml of undiluted hydrochloric acid and heat at 100 °C for 1 hour.
  (*g*) Filter through filter paper and adjust the pH to 7.8. Keep overnight at 4 °C.
  (*h*) Sterilize by autoclaving at 121 °C for 30 minutes.
— Hyaluronidase:
  (*a*) Inoculate 1 litre of Hartley broth containing 2% inactivated horse serum with 5 ml of an 8-hour culture of *Streptococcus pyogenes* (group A) strain A4NW Kalbak. Incubate at 37 °C overnight. Collect the supernatant by filtration through a bacterial filter.
  (*b*) To the filtrate add ammonium sulfate to 57% saturation (430 g/1000 ml) with stirring and allow the precipitate to form at 4 °C overnight.
  (*c*) Separate the precipitate by centrifugation (approximately 1500*g* for 15 minutes) and dissolve it in distilled water (approximately 5% of the original volume); dialyse against distilled water at 4 °C (changed two to three times) to remove ammonium ions (e.g. test with Nessler reagent).
  (*d*) Distribute 10-ml aliquots into test tubes and store at –30 °C
— Potassium hyaluronate substrate. Dissolve potassium hyaluronate (commercially available) in buffer (1) in a concentration of 1 mg/1.5 ml.
— Acidified horse serum. Dilute sterile horse serum 1:10 with buffer (2) and adjust the pH to 3.1 with 4 mol/l hydrochloric acid. Heat in a boiling water-bath for 10 minutes. Cool and store at 4 °C for at least 24 hours before use. The preparation remains stable for about 1 month.

Method

1. Dilute the hyaluronidase in geometric series in six auxiliary test-tubes. To the first tube add 0.75 ml and to the remainder 0.5 ml of buffer (1). Add 0.75 ml of hyaluronidase to the first tube and after mixing transfer 1 ml into the following tube and so on to the sixth.
2. Prepare another set of eight test-tubes. Transfer 0.2 ml of each hyaluronidase dilution into the corresponding six test-tubes containing 0.5 ml of buffer (1) as shown in Table 12.
3. Add 0.3 ml of substrate solution to each of the six tubes.
4. Prepare the following controls:
   Control 1: 0.7 ml of buffer (1) and 0.3 ml of substrate
   Control 2: 0.85 ml of buffer (1) and 0.15 ml of substrate
5. Mix well and incubate all tubes at 37 °C for 30 minutes.

Table 12. Dilution scheme for the titration of hyaluronidase

	Test-tube no.							
	1	2	3	4	5	6	Control 1	Control 2
Buffer (1) (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.85
Hyaluronidase (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0	0
diluted to[a]	1:2	1:3	1:4.5	1:7	1:10	1:15	—	—
Substrate (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.15

[a]Dilution in buffer (1); final content of the test-tubes for incubation at 37 °C (step 5).

6. After incubation inactivate the remaining enzyme in a water-bath at 60 °C for 10 minutes.
7. Add 3 ml of buffer (2) to each tube.
8. Add 1 ml of acidified horse serum to each tube.
9. Mix and leave the tubes at room temperature for 30 minutes.
10. Measure the transmittance with a spectrophotometer at 580 nm. The hyaluronidase titre, expressed in turbidity reducing units (TRU), is equal to the highest dilution of enzyme that decreases the turbidity caused by 0.2 mg of substrate (control 1) to that caused by 0.1 mg (control 2). If the difference in transmittance of the two controls does not reach at least 15% use another batch of potassium hyaluronate.

12.3 Determination of anti-hyaluronidase in human sera

Equipment and supplies

— Water-baths at 37 °C, 56 °C and 60 °C
— Test-tubes
— Spectrophotometer
— Pipettes
— Serum samples to be evaluated

Reagents (see section 12.2)

— Buffer (1)
— Buffer (2)
— Hyaluronidase
— Potassium hyaluronate substrate
— Acidified horse serum

Method

1. Inactivate the serum samples at 56 °C for 30 minutes.
2. Dilute the inactivated serum in a geometric series as follows: to each of six test-tubes add 0.5 ml of buffer (1). In an auxiliary test-tube prepare serum dilution 1:50 and transfer 0.5 ml into the first test-tube (1:100). Stir and transfer 0.5 ml into the second and so on until the sixth (1:3200) from which 0.5 ml is then discarded (Table 13).
3. Dilute streptococcal hyaluronidase with buffer (1) to obtain 15 TRU in 1 ml. To each test-tube add 0.2 ml containing 3 TRU.
4. Mix and incubate for 15 minutes at room temperature.
5. Add 0.3 ml of substrate solution (containing 0.2 mg) to each test-tube.

Table 13. Dilution scheme for the determination of anti-hyaluronidase titre in human sera

	Test-tube no.							
	1	2	3	4	5	6	Control 1	Control 2
Serum (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0	0
diluted[a] to	1:100	1:200	1:400	1:800	1:1600	1:3200	—	—
Hyaluronidase (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0	0
Substrate (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.15
Buffer (1) (ml)	0	0	0	0	0	0	0.7	0.85

[a] Dilution in buffer (1); final content of the test-tubes for incubation at 37 °C (step 7).

6. Prepare the controls as follows (Table 13).
   Control 1: 0.3 ml of substrate (containing 0.2 mg of potassium hyaluronate) and 0.7 ml of buffer (1).
   Control 2: 0.15 ml of substrate (containing 0.1 mg of potassium hyaluronate) and 0.85 ml of buffer (1).
7. Mix well and incubate in a water-bath at 37 °C for 30 minutes.
8. Immediately after incubation transfer the test-tubes to a water-bath at 60 °C for 10 minutes to inactivate the remaining enzyme.
9. Add 3 ml of buffer (2) and 1 ml of acidified horse serum to each test-tube.
10. Mix and incubate at room temperature for 30 minutes.
11. Measure the turbidity using a spectrophotometer at 580 nm.
12. The anti-hyaluronidase titre is determined by interpolation. It is expressed by the serum dilution with a transmittance equal to the transmittance of control 2.

13. Indirect bactericidal test for measurement of anti-M antibodies

13.1 Introduction

13.1.1 Principle of the test

Streptococcal cells are protected by M protein against phagocytosis; streptococci possessing sufficient quantities of M protein grow freely in "normal" human blood, while those that do not are killed. *In vitro*, streptococcal strains that possess M protein (M+) are phagocytosed and killed by human polymorphonuclear leukocytes only in the presence of homologous anti-M antibody. If type-specific anti-M antibody is absent or if only heterologous anti-M antibodies are present, M+ streptococci propagate readily in fresh human blood. The antiphagocytic effect of streptococcal M protein and its neutralization by type-specific antibody form the basis of the bactericidal test.

Two main types of bactericidal test are used in the streptococcal research laboratory. The *indirect bactericidal test* measures survival of streptococci in human blood using blood obtained from a donor lacking antibody to the test strain and antiserum procured from another source. The antiserum used may be from a human source (i.e. a patient being screened for antibody following a streptococcal infection) or may be a hyperimmune animal serum. Perhaps the most common use of the indirect bactericidal test is to determine the type-specific anti-M antibody content of a serum. In this test, a measured quantity of M+ streptococci is incubated in freshly drawn human blood together with serum that contains or is to be tested for the presence of anti-M antibody. Presence of the antibody is determined by the degree to which the streptococcal strain is phagocytosed and killed in the presence of the serum. The indirect bactericidal test can also be used to identify or verify the M type of a strain by determining the extent to which it is killed in the presence of a known type-specific antiserum.

Less commonly used today is the *direct bactericidal test*, in which no additional antiserum is added to the system: the blood donor is the source of anti-M antibody, if any. This test has been used to verify the development of protective anti-M antibodies in a patient following a documented streptococcal infection. One variation of the direct bactericidal test is often referred to as a "blood rotation". This utilizes a donor prescreened and shown to lack antibody to the strain(s) being tested; no additional antibody is added. The test is used to assess the M protein content of a strain by determining the ability of the strain to grow in human blood in the absence of type-specific anti-M antibody. This is very important, for example, in evaluation of the suitability of a strain for use in production of anti-M sera. This test has also been described as a means of enhancing the M protein content of a strain that exhibits poor growth in normal human blood. Colonies surviving blood rotation should, in theory, be richer in M protein than the original inoculum composed of both survivors and non-survivors (*131, 132*).

The bactericidal test must be carried out in conditions such that: streptococci grow readily unless prevented by specific antibody; microphages are active and able to engulf and kill the M-negative or antibody-coated streptococci; and opportunity for streptococcus–phagocyte contact occurs throughout the incubation. After completion of the test, the quantity of viable streptococci in

the sample is measured and compared with the number inoculated at the start of the test (*133–135*).

13.1.2 Test components

Three components are necessary for the indirect bactericidal test: streptococci; sera containing or to be tested for type-specific antibody; and freshly drawn human blood. Serum to be tested is obtained from a human or animal source and added to the system. The human blood is obtained from a normal adult donor, prescreened and shown to lack antibodies to the serotype(s) being tested and to produce an acceptable bactericidal effect.

Streptococci

A measured quantity of M+ streptococci in the logarithmic growth phase is normally used as inoculum. Indirect bactericidal tests utilizing stationary phase cells have also been described but are less commonly used.

Human or hyperimmune animal sera

The crucial elements in selecting sera to be used in bactericidal tests are M-antibody content and the absence of constituents that might inhibit streptococcal growth or interfere with leukocyte activity. The sera must not be contaminated and must not contain antibiotics or other bactericidal or bacteriostatic agents in concentrations that would inhibit the growth of streptococci. In this test system, final concentrations of thiomersal (thimerosal USP) of up to 1:10000 or of sodium azide of up to 1:1000 are acceptable. Sera with gross haemolysis are unsuitable. Long-term storage of sera is best accomplished by freezing (without any preservative) at –20 °C or, preferably, –70 °C. Rapid freezing and thawing help maintain sera in good condition. However, repeated freezing and thawing of the samples gradually lowers the antibody titre. Some rabbit sera have to be diluted at least 1:3 with physiological saline or heated at 62 °C for 30 minutes to display the full effect of their anti-M antibodies. Some investigators routinely heat-inactivate all sera (at 56 °C or 62 °C for 30 minutes) before testing in order to eliminate the variable and unpredictable nonspecific effects of thermolabile serum components. In the experience of the Prague laboratory, there is no need for such routine heat-inactivation before titration. Sera are normally used unabsorbed in the test, although absorbed sera can also be used.

Human blood

Human blood from normal adults is used. Any blood group is satisfactory for tests performed with animal sera. Blood group O should be used when examining human sera, to avoid incompatibility. The blood must not contain antibiotics or anti-M antibodies to the streptococcal types being employed; heterologous anti-M antibodies do not interfere (Table 14). Blood should be drawn immediately before use and should not be used if more than 2 hours have elapsed. Heparin at a concentration of 8–10 units/ml of blood is used as the anticoagulant. (High concentrations of heparin substantially reduce the bactericidal activity of the system.)

Under favourable conditions (tests with sera possessing a high anti-M antibody titre and with strains that do not grow excessively fast) an acceptable bactericidal effect may be obtained with most human bloods. However, in the

Table 14. Example of the preliminary selection of a suitable bactericidal system

	Group A streptococcal strain M type					
	6		12		49	
Inoculum:						
CFU in individual drops (0.02 ml)	71	13	58	13	114	21
	66	14	71	9	119	21
	61	9	69	11	109	16
CFU/0.04 ml (average)	132	24	132	22	228	39
	Blood donor BM					
Growth[a] in the presence of:						
— normal rabbit serum (CFU/0.1 ml)	18	2	23000	2800	18100	3520
— homologous antiserum (CFU/0.1 ml)	0	0	14	1	9	0
Growth index[b]	<1		167		81	
Bactericidal index[b]	NE		1720		2402	
	Blood donor KZ					
Growth in the presence of:						
— normal rabbit serum (CFU/0.1 ml)	14700	3250	2	0	13700	2480
— homologous antiserum (CFU/1.0 ml)	1	0	0	0	20	0
Growth index	115		<1		61	
Bactericidal index	17950		NE		809	
	Blood donor SZ					
Growth in the presence of:						
— normal rabbit serum (CFU/0.1 ml)	14700	3900	14700	2240	7040	1520
— homologous antiserum (CFU/0.1 ml)	1	0	2	0	0	0
Growth index	119		110		32	
Bactericidal index	18600		8470		>8560	

CFU, colony forming units; NE, not evaluated

[a] Growth after 3 hours in roller.

[b] For calculation of the growth index and bactericidal index see section 13.2.2.

experience of the Prague laboratory, only a few donors among dozens examined have proved suitable for the detection of low levels of antibody (Table 15). The adequacy or inadequacy of human blood for bactericidal tests is a relatively stable characteristic of individual donors over many years (*135*).

13.1.3 Equipment

Glassware quality and volume measurement

Cleanliness of test-tubes and other glassware used in the test must be maintained at the standard required for tissue culture. All test components must be accurately measured using calibrated dropping pipettes, serological pipettes or mechanical micropipettes.

Table 15. Example of the selection of blood donors: "sensitivity" of bactericidal systems

	Blood donor					
	FS		SJ		HA	
Inoculum PT '3800" (CFU/tube)	86	86	86	86	86	86
Growth[a] with normal rabbit serum (CFU/0.1 ml)	3970	3200	3200	2880	3520	4400
Growth with rabbit anti-M "3800" antiserum (CFU/0.1 ml):						
— Undiluted	0	0	0	0	11	5
— Diluted 1:3	0	0	0	0	600	580
— Diluted 1:9	0	0	21	19	2170	1680
Growth index[b]	42		35		46	
Bactericidal index[b] with anti-M serum:						
— Undiluted	>7170 = >4+		>6080 = >4+		495 = 3+	
— Diluted 1:3	>7170 = >4+		6080 = >4+		7 = 0	
— Diluted 1:9	>7170 = >4+		152 = 2+		2 = 0	

[a] Growth after 3 hours in roller.

[b] For calculation of the growth index and bactericidal index see section 13.2.2.

Rotation device

Successful performance of the bactericidal test requires a device for rotating or rolling the tubes to maintain blood cells and streptococci in homogeneous suspension throughout the 3-hour incubation. This is satisfactorily accomplished by a roller at 8 rpm, rotating the test tubes either end-over-end or, provided that the axis inclination is close to the horizontal plane, parallel to the drum axis.

13.1.4 Evaluation of test results

Bactericidal test results are evaluated by comparing the change in colony count during the 3-hour rotation in the presence of both serum lacking type-specific antibody and the test serum. Growth of the strain in human blood is measured by the growth index. Although the method for calculating the growth index may vary in detail from laboratory to laboratory (see sections 13.2.2 and 13.3.3), in all cases a larger value indicates greater growth in the test system. There is no right or wrong way to calculate the growth index of a strain being evaluated in a bactericidal test; the method that best suits the needs of the investigator should be used. However, index values alone are meaningless unless the test protocol and the calculation method are documented. The bactericidal effect of an antiserum is measured by the bactericidal index. Again, calculation methods may vary in small details, but regardless of the method (assuming the growth index is sufficient), a larger value indicates a greater bactericidal effect, i.e. greater killing of the streptococci by phagocytes as a result of the opsonic activity of type-specific antibody.

13.1.5 Selection of suitable bactericidal systems

Before a set of indirect bactericidal tests is started, blood from a number of donors should be tested with several different strains of the particular streptococcal M types being evaluated. Careful studies carried out in the Prague laboratory demonstrate the importance of this testing (see section 13.2 for the methodology). The test shown (Table 14) utilized three blood donors and three different streptococcal M types and yielded the following information. (1) All three streptococcal strains tested are suitable for bactericidal testing (all grew rapidly in at least one donor's blood). (2) Blood donor BM cannot be used for type 6, and donor KZ cannot be used for type 12. Blood donor SZ appears to be acceptable for all three types.

In general, if a strain does not grow in any of the bloods used, it probably lacks sufficient M protein for use in bactericidal tests. Specific growth inhibition of a given strain by a blood donor's antibodies will usually result in the strain growing well in a least one other donor's blood and, simultaneously, the inhibiting blood will allow good growth of at least one other streptococcal strain of a different M type.

A more exact basis for the selection of suitable blood donors and a classification of their "bactericidal sensitivity" is provided by examining the bactericidal effect produced by a serially diluted anti-M serum in a reference system. An example of such a test carried out in the Prague laboratory is shown in Table 15. In this case the bactericidal effectiveness of three blood donors was tested against a single strain (a provisional type PT "3800") used as a uniform inoculum of 86 colony-forming units (CFU) for each test-tube throughout. The results clearly demonstrate that blood donors may differ significantly in their bactericidal effectiveness in spite of a comparable strong response to undiluted high-titre antiserum. Donor HA is unsuitable for the detection of low levels of anti-M antibodies as shown in the test by the loss of acceptable bactericidal response to the reference anti-M serum when only slightly diluted (1:3).

To minimize possible encounter with homologous anti-M antibodies to the inoculum employed, a streptococcal M type of rare occurrence is preferred in such "sensitivity" testing. The relative bactericidal effectiveness of blood from a given donor as characterized by a single system, e.g. M "3800"–anti-M "3800", is representative of bactericidal effectiveness for strains of other M types as well and does not change significantly over time. The combination of strains and donor(s) that provides the best streptococcal growth and the best growth inhibition in the presence of diluted homologous antiserum is then selected for the final tests.

13.1.6 Bactericidal test controls

Each test must include the following controls, which are essential for the accurate interpretation of results.

1. Control to verify the ability of each strain used in the test to propagate in the particular blood. A sample composition would be: normal rabbit serum + streptococcal inoculum + human blood in volumes identical to those used in the actual test. As a rule, this control represents the maximum growth of the inoculum attainable in the given system.
2. Control for bactericidal efficiency of the blood in the presence of homologous anti-M antibodies. A sample composition would be: homologous hyperimmune serum suitably diluted + streptococcal inoculum + human

blood. Again, volumes and conditions must exactly match those of the actual test. If reference homologous anti-M sera are not available for the strains under study, an indirect control in the form of some other already tested streptococcal strain of different type and corresponding antiserum should be included. Evidence of good bactericidal activity, even against a single type, is an important characteristic of the blood even in relation to other streptococcal types. If new blood donors who have not been tested with the particular M type(s) are being used, it is reasonable to test several of the sera in parallel in two different bloods.

3. Control for type specificity of bactericidal activity. Each serum being tested should be examined against streptococcal strains of at least two different M types. Inhibition of one type by the serum and unimpaired growth of the other type is evidence of specific inhibition. Day-to-day variation is inherent in a test of this complexity, and if quantitative documentation of a change in titre in an individual is required, it is essential to examine all sera from that individual simultaneously in the same test.

13.2 Bactericidal test method 1

13.2.1 Procedure

It should be noted that the general principles outlined in section 13.1 apply throughout section 13.2. Important additional methodological details specific to the test as performed in the Prague laboratory are presented in the following sections.

Glassware, volume measurement and incubation in roller

Inocula, sera and blood are pipetted drop-wise directly onto the bottom of test-tubes (14 × 120 mm) without touching their walls. Sera and inocula are measured by means of dropping pipettes calibrated mostly for a drop size of 0.02 ml. If another drop volume is used, a corresponding change in the final dilution is necessary to obtain a suitable CFU count.

Streptococcal inoculum

For immediate use

Centrifuge (at 1500*g* for 10 minutes) an overnight 6-ml serum broth culture (serum broth = Todd–Hewitt broth with 20% added normal calf or horse serum) and resuspend the pellet in 0.8 ml of fresh serum broth. Transfer a sufficient amount of this suspension to 6 ml of serum broth to depress the transmittance (at 560 nm) to 70% of a serum broth blank. Incubate the resulting suspension at 37 °C in a water-bath for 90 minutes and then dilute in Todd–Hewitt broth. The dilution required may be different for each strain and is predetermined in preliminary growth experiments. The dilution steps are between 1:4 and 1:7 (e.g. 10^{-4}, $10^{-4}/4$ and $10^{-4}/16$, or 10^{-4}, $10^{-4}/7$ and $10^{-4}/49$). The objective is to obtain 20–40 colony forming units (CFU) per 0.04 ml at the highest dilution. At least two or three different inocula are prepared with each strain (Table 14). It would be risky to use only a single dilution for each strain since the standardization employed does not always give inocula with the desired CFU count.

Deep frozen, ready for use

Preparation of standardized inocula stored at –70 °C is the same as for freshly prepared inocula except that the final culture dilutions to be frozen are pre-

pared in serum broth. Aliquots (0.5–1.0 ml) of 10^{-5} and 10^{-4} dilutions are rapidly frozen in an ethanol/dry-ice (CO_2) bath and transferred (on dry ice) to a deep-freeze unit at –70 °C for storage. Immediately before use, thaw the inoculum *rapidly* (by transferring the test-tube(s) from –70 °C directly into a water-bath at 25–37 °C), dilute with serum broth if necessary, and pipette immediately into tubes for the test. As a rule, the final culture dilution (= inoculum) should contain 50–100 CFU in 0.04 ml. (The highest sensitivity of the bactericidal test is achieved with relatively small inocula not yet interfering with the reproducibility of test results.) A single inoculum is used for each strain and each serum is tested in two parallel test-tubes (Table 14). Even after storage for several years, correctly prepared and preserved frozen inocula give the same results in bactericidal tests as freshly prepared inocula. The rapidity of freezing and thawing as well as the constant low temperature (–70 °C) seem to be critical for adequate results (*136*).

A preliminary check to determine the CFU count per 0.04 ml of inoculum should be performed before a suspension is frozen. Apply 3–4 separate drops (0.02 ml each) of each culture dilution to be frozen to a blood agar plate. The inoculum is calculated as the arithmetic average of the number of colonies grown from 0.04 ml of the suspension. The same method is used to check the inoculum size in the actual bactericidal test. With strains forming mucoid confluent colonies, the pour plate method (see below) is used to check the CFU count.

Test mixture for incubation in roller

First pipette serum into test-tubes. For rabbit serum (normal or hyperimmune, the latter diluted with saline if necessary), a volume of 0.02 ml is optimal. For human serum (as a rule undiluted), use a volume of 0.06 ml. Add 0.04 ml of the streptococcal culture dilution (the inoculum). Then add 0.30 ml of normal human blood to each tube. Heparin (5000 IU/ml; working solution 200 IU/ml in phosphate-buffered saline) at a concentration of 8 IU/ml of blood, is used as the anticoagulant. Seal the test tubes *firmly* with tight rubber stoppers and incubate at 37 °C in the roller at 8 rpm for 3 hours. The tubes are rotated parallel to the drum axis, the axis inclination to horizontal plane being 10–15°. The small differences in total volume between test-tubes containing rabbit or human sera proved to be negligible for test results and need not be adjusted.

Pour plates

The bactericidal test result is determined by culturing and counting the number of streptococcal CFU surviving the test. Stop the incubation by placing the roller drum with test-tubes in an ice-water-bath. After thorough mixing, withdraw 0.1 ml with a micropipette from each test-tube and transfer to a Petri dish (diameter 9 cm) containing approximately 2.5 ml of Todd–Hewitt broth. Immediately add 15 ml of freshly prepared blood agar containing 2.5% of defibrinated sheep blood (cooled to 48–50 °C) and mix the contents thoroughly to achieve even colony distribution throughout. Incubate the pour plates overnight at 37 °C.

Colony counting in pour plates

The number of CFU surviving the bactericidal test is established by colony count. The counting is conveniently performed against a grid with the help of a magnifying lens and a mechanical counter. Streptococcal colonies are

counted in selected grid sectors or squares, depending on colony density, and the result is calculated for the entire plate area. Generally, of the total, not more than 300–350 CFU per plate are counted. Pour plates prepared with diluted samples do not substantially improve colony counting results.

13.2.2 Reading and evaluating results

Growth index

The growth index indicates the degree of strain growth in non-immune blood in the absence of antibody. The growth index is calculated using the following formula:

$$\text{Growth index} = \frac{\text{CFU count per 0.1 ml of sample with normal serum after 3 hours in roller}}{\text{number of CFU inoculated}}$$

If an inoculum is used at three different dilutions, the numerator will be the sum of colonies in all three pour plates with normal serum and the denominator will be the sum of CFU inoculated into the three samples. An example for two inoculum dilutions is given in Table 14 and an example for duplicate samples with identical inoculum in Table 15.

The lower growth index limit for strains suitable for anti-M antibody titration is about 30 (which, for a test-tube content of 0.36 ml, corresponds to an inoculum multiplication of 108 times, or roughly 7 generations). With a growth index of <20 the reproducibility of test results deteriorates, probably due to the increased impact of non-type-specific factors. Interpretation of such results is much less clear than that of results obtained with strains of the same type propagating at a higher rate with a growth index of between 30 and 130. At the other extreme, strains with a growth index of >140 (more than 9 generations) are less sensitive to low antibody titres.

Bactericidal index

The bactericidal index expresses the degree of inhibition of streptococcal growth due to antibody and is expressed as a ratio of the final CFU count in the absence of antibody to the CFU count in the presence of the test serum (*135*):

$$\text{Bactericidal index} = \frac{\text{CFU count per 0.1 ml of sample with normal serum after 3 hours in roller}}{\text{CFU count per 0.1 ml of sample with serum under test (immune) after 3 hours in roller}}$$

If a uniform inoculum is used and titration is performed in two parallel samples, the numerator will be the sum of colonies in both pour plates with normal serum and the denominator will be the sum of colonies in both pour plates for samples with identical antiserum.

The Stollerman scale is used to express bactericidal index values (see Table 16).

In the range of the lowest degree of inhibition (+/–) the bactericidal index value (e.g. 32) signifies growth inhibition by a 5-generation factor as compared with the normal serum control. For example, for an inoculum size of

Table 16. The Stollerman scale for expressing bactericidal index values (*123*)

Bactericidal index	Score
<25	0
25–50	+/–
51–100	1+
101–200	2+
201–500	3+
>500	4+

100, a growth index of 64, and a count of surviving CFU in 0.1 ml of sample with antiserum of 200:

$$\text{Bactericidal index} = \frac{6400}{200} = 32$$

The count of the surviving CFU in the normal serum control is thus 32 times higher than in the test sample ($32 = 2^x$; $x = 5$).

The bactericidal index is not equivalent to the antibody titre. A more precise estimation of anti-M antibodies in serum samples is obtained by examining serial serum dilutions for a threshold bactericidal index of 25. The entire Stollerman scale usually covers only a rather narrow range of titres. The transition from strong positivity (4+) to negative (bactericidal index <25) is often abrupt (in 75% of instances, in serial dilutions with a quotient of 1:3, such a sudden titre drop was observed within one or two dilution steps only — see Table 15). Very strong reference rabbit anti-M sera are highly efficient even in a dilution of 1:100 or higher with suitable blood donors.

The bactericidal index provides a rather broad characteristic of a particular serum and is especially useful for comparing the bactericidal activity of sera examined simultaneously in the same test. Indices from different tests are comparable only within certain limits. They are not reproducible in detail, even if the same blood donor is used. Use of identical inocula (deep-frozen ready-for-use aliquots) and identical reference sera in dozens of successive tests revealed the dominant role in the variability of test results of the individual human blood samples that have to be taken freshly for each test. The bactericidal index value is strongly dependent on inoculum size and strain growth index, especially if these deviate beyond the optimal limits. Strongly positive results remain strongly positive and negative results are always negative in the blood of the same donor. Variation occurs most frequently with weak bactericidal index values. Such values (around 25 or lower) invite repetition of the test, if possible in a blood known to produce a good bactericidal effect even with low levels of anti-M antibodies (Table 15).

13.3 Bactericidal test method 2

Many methods have been described for performing the indirect bactericidal test. The principles outlined in section 13.1 apply throughout this section. Details specific to the test(s) as performed in the Minneapolis laboratory are presented below. The Minneapolis laboratory uses two variations of the test that differ in some details from the methods used in the Prague laboratory. However, even though the details may differ, the very important principles

outlined in the methods and discussions above must be adhered to. This is an extremely difficult test to perform well and reproducibly. Utmost attention must be paid to the smallest details and absolute consistency must be maintained with regard to the way each sample is handled within a test and the way the test is performed day-to-day.

13.3.1 Lancefield method as used in Minneapolis

Lancefield's basic bactericidal test method (*133*, *137*), like the procedure used in Prague, uses a logarithmic growth phase inoculum. The inoculum is prepared by growing the strain overnight in Todd–Hewitt blood broth (containing an additional 1% neopeptone) and then transferring 1 ml of this overnight culture into 4 ml of warm Todd–Hewitt broth + 1% neopeptone without blood. This culture is then grown at 35–37 °C for 2 hours (logarithmic growth phase). If the culture tends to grow in a "granular" or "clumpy" manner, cultivation of the strain with a small amount of serum added will often alleviate this problem. The culture is then diluted in Todd–Hewitt broth for use in the test.

Lancefield commonly included four fourfold streptococcal dilutions in her tests (usually 10^{-5}, $10^{-5}/4$, $10^{-5}/16$, $10^{-5}/64$). If laboratory technique is such that reproducible results can be obtained, testing with a single dilution may be adequate. If a single dilution is used, it should contain approximately 100–200 CFU/0.1 ml. The dilution required to prepare the test inoculum is predetermined by plate count in preliminary experiments that duplicate exactly the inoculation and growth conditions of the actual test. If the conditions are duplicated with accuracy, these predetermined dilutions will result in CFU counts very close to those obtained in the preliminary test.

In a preliminary test to predetermine dilution, 0.1-ml aliquots of 10^{-4}, 10^{-5} and 10^{-6} dilutions of the 2-hour culture are plated. After counting, the actual dilutions to be used in the test are determined by interpolation. Testing duplicates of a single dilution will increase accuracy and allow assessment of consistency. Once prepared, the dilutions are kept on ice until completion of the test.

Use of controls is as described in section 13.1.6. The test is performed in small glass test-tubes (10 or 12 × 75 mm) with tightly sealing rubber stoppers or in polystyrene plastic snap-top tubes (12 × 75 mm). Scrupulous attention must be paid to the cleanliness of all glassware and equipment that comes into contact with any of the test components. Human blood for use in the test is selected as described in section 13.1.5. Heparin sodium is used as the anticoagulant, at a final concentration of 10 IU/ml of blood. High concentrations of heparin must be avoided.

Test components are added to the tubes in the following order and volumes:

antiserum (human or hyperimmune animal)	0.05 ml
dilution of logarithmic growth phase streptococcal culture	0.10 ml
normal human blood	0.30 ml
total volume	0.45 ml

To maximize contact of the various test components, the tubes are rotated end-over-end at 8 rpm for 3 hours at 35–37 °C. At the time the 0.1-ml logarithmic growth phase culture dilution is added to the tube to be rotated, a second 0.1-ml aliquot is removed for a plate count to determine the number of CFU added to the system. The plate count is done as a pour plate by adding the

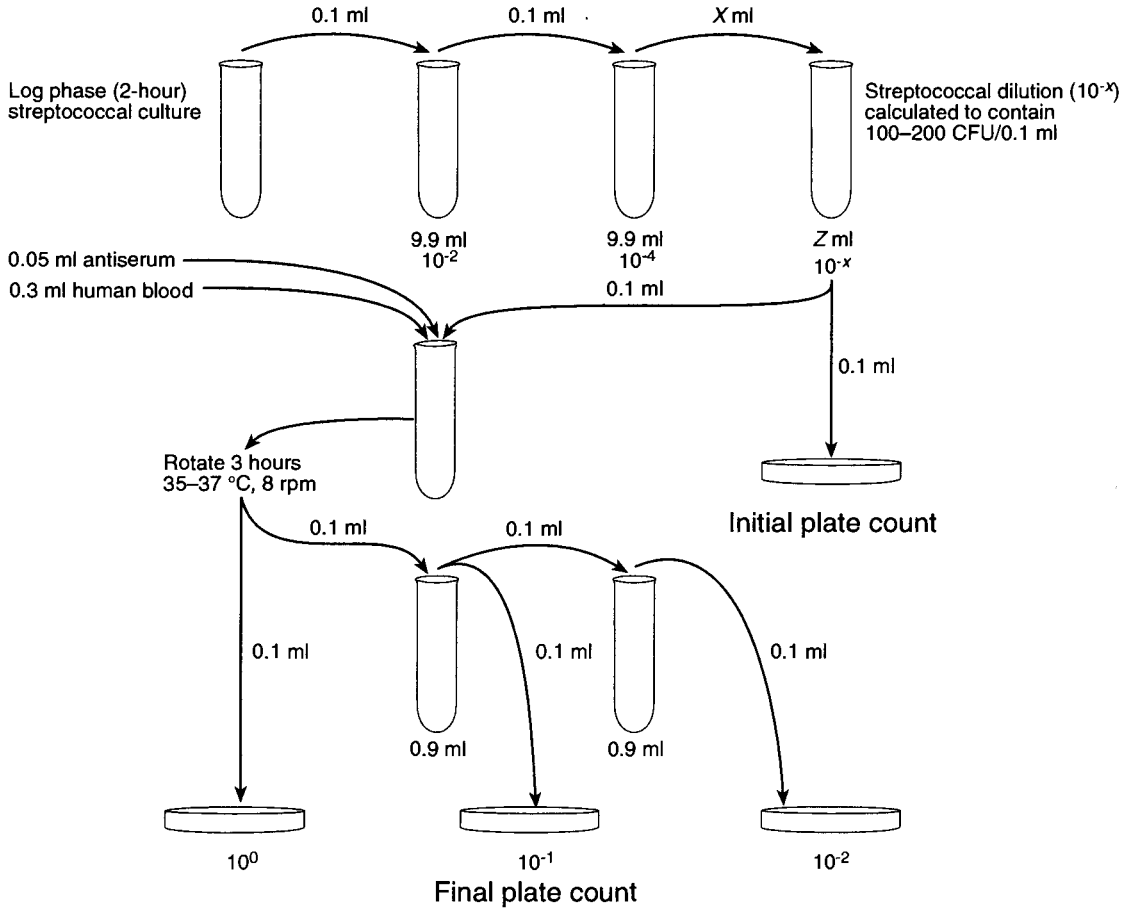

Fig. 13. Flow diagram for the indirect bactericidal test

0.1-ml aliquot to 0.5–1 ml of saline broth (5 ml Todd–Hewitt broth in 100 ml saline) contained in a standard Petri dish (100 × 15 mm). The pour plate is then prepared using 12 ml of blood agar base (cooled to 48–50 °C) and 0.3 ml of sheep blood. Similarly, at the end of the test, 0.1 ml is removed from the tube and plated to determine the number of streptococcal CFU surviving the test. The Minneapolis laboratory generally prefers to make and plate dilutions of the test mixture after rotation. Dilutions of 10^{-1} and 10^{-2}, plated in addition to the undiluted sample, will usually give at least one plate in the optimal countable range of 30–300 CFU/plate. Growth index and bactericidal index are calculated as described in sections 13.3.3 and 13.3.4. A flow diagram for the indirect bactericidal test as described above is shown in Fig. 13.

13.3.2 Preopsonization bactericidal test method

A variation of the bactericidal test used in the Minneapolis laboratory was developed to improve the sensitivity of the test to the low titres of antibody often encountered in human sera (*138*). It involves two basic changes. First, stationary phase rather than logarithmic growth phase cells are used for the inoculum in the test, in order to reduce the initial growth rate of streptococci and thereby increase the opportunity for phagocytosis to occur. The second modification is the incorporation of a preopsonization step. Preopsonization, a concept originally described by Beachey & Cunningham (*139*), refers to premixing and preincubating the antiserum together with the streptococci. This allows antibody, if present, to bind to the streptococci before they are exposed to the phagocytes, thus increasing the sensitivity of the system.

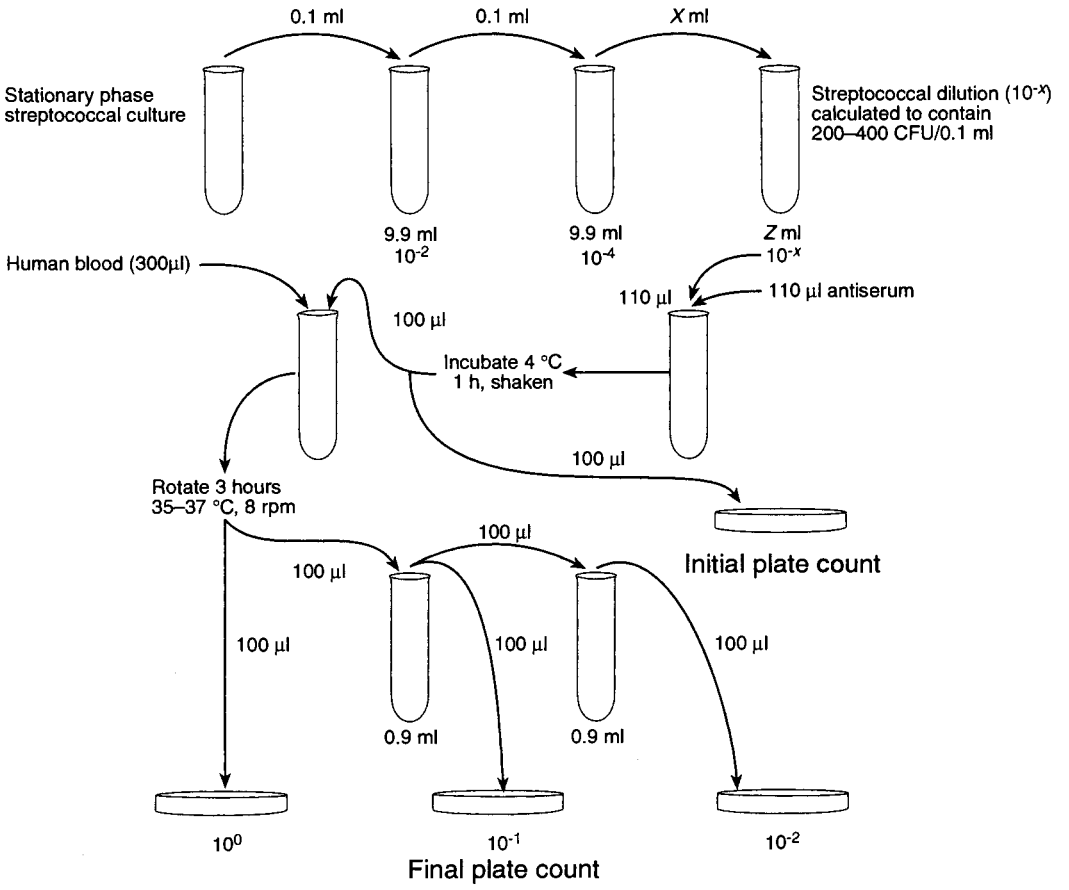

Fig. 14. Flow diagram for the preopsonization indirect bactericidal test

In practice, the preopsonization bactericidal test is performed as follows. Antiserum in a volume of 110 µl is mixed with 110 µl of a dilution of stationary phase streptococci, and this mixture is then incubated with gentle shaking at 4 °C for 1 hour. After this preopsonization step, 100 µl of the mixture is removed and added to 300 µl of heparinized normal human blood and rotated end-over-end for 3 hours as in a normal bactericidal test. A second 100-µl aliquot of the preopsonization mixture is plated to determine initial inoculum size. The remainder of the test is done as for the standard Lancefield method described above. A flow diagram for the preopsonization indirect bactericidal test is shown in Fig. 14.

13.3.3 Reading and evaluating results

Growth index

It should be noted that there is no established and universally accepted definition for growth index. The Minneapolis laboratory prefers to base its definition on the principles described by Top et al. (*137*), in accordance with which the growth index represents the actual total amount of growth that occurs during the test and corresponds to what is referred to as the "inoculum multiplication" in section 13.2.2. The growth index is not then influenced by differences in test protocol, such as changes in the total volume of the test mixture or the volumes plated; it is calculated as follows:

$$\text{Growth index} = \frac{\text{calculated CFU count in the total sample volume after 3-hour rotation}}{\text{number of CFU inoculated into sample at beginning of test}}$$

Dilution occurs during the test, the dilution factor depending on the volumes of the various components used. This dilution should be taken into account when growth index is calculated. The total volume in the Lancefield bactericidal test above is 0.45 ml and in the preopsonization method, 0.4 ml. The 0.1-ml volume of streptococci added initially is distributed throughout the entire test volume. When the 0.1-ml aliquot is removed for plating at the end of the test, this represents only a fraction of the total volume. The CFU count after rotation must therefore be multiplied by the reciprocal of the fraction, 4.5 or 4.0 in the examples given, to be strictly comparable with the initial count (*137*). If dilutions of the sample after rotation are plated as described above, this dilution is also included in the calculation. The growth index for the Lancefield method described above would therefore be calculated as follows:

$$\text{Growth index} = \frac{\text{CFU in 0.1 ml of final sample dilution} \times 4.5 \times \text{final plate dilution factor}}{\text{number of CFU inoculated into sample at beginning of test}}$$

For example, if the initial 0.1 ml inoculum gave a plate count of 100 CFU and, after rotation, a 10^{-2} dilution of the test mixture contained 100 CFU/0.1 ml, the growth index would be calculated as follows:

$$\text{Growth index} = \frac{100 \times 4.5 \times 100}{100} = 450$$

It should also be noted that calculations of growth index using this system are not limited to growth in normal serum but are also used for tests with type-specific serum. In some circumstances it may be of interest to know the ability of a strain to grow in homologous antiserum in absolute terms as well as in relative terms as expressed in the bactericidal index (described below). Using the methods described in section 13.3, the Minneapolis laboratory considers a growth index of 32 ($\log_2 = 5$) to be the minimum acceptable for a strain being tested with a suitable blood donor in the absence of type-specific antibody. Below this level the strain probably does not possess enough M protein for reliable testing.

Bactericidal index

The bactericidal index can be thought of as the ratio of the growth index (as calculated above) in normal serum to the growth index in type-specific antibody:

$$\text{Bactericidal index} = \frac{\text{growth index in normal serum}}{\text{growth index in type-specific antiserum}}$$

For most studies a bactericidal index of 32 is the minimum considered significant.

13.3.4 Expressing growth and bactericidal index values as logarithms

When the results of bactericidal tests are analysed — expressed either by the growth index or the bactericidal index — use of logarithms to the base 2 ($\log_2$) rather than the actual numbers obtained from the calculation has several advantages. First, this value can be thought of intuitively in terms of generations of growth, even though for streptococci, which are chaining organisms, this is not strictly accurate. Second, it is easier to apply statistical tests to the

results, since use of logarithms reduces the misleadingly high growth index values obtained with very rapidly growing strains and the high bactericidal index values seen with strains that are almost completely killed. This is also accomplished semiquantitatively by the Stollerman scale (section 13.2.2), but use of $\log_2$ values allows mathematical comparisons of results. Logarithms to the base 2 can be easily computed on simple calculators using the following formula:

$$\log_2 X = \frac{\log_{10} X}{\log_{10} 2} = \frac{\log_{10} X}{0.301}$$

The growth index of 450 obtained in the preceding example would therefore be expressed as a $\log_2$ as follows:

$$\log_2 450 = \frac{\log_{10} 450}{0.301} = \frac{2.653}{0.301} = 8.8$$

Thus, this strain grew through nearly nine generations during the 3-hour rotation.

13.4 M serotyping by means of the bactericidal test

The indirect bactericidal test, used primarily for the detection of protective, type-specific anti-M antibodies, can also be used as the reference method for verifying the M type of group A streptococci (*133*). This is a difficult and time-consuming test and would be used only in very limited circumstances such as verification of new M types or clarification of conflicting or unexpected results obtained in routine typing tests. The test is not appropriate for routine use.

The organization of the test is the same as for antibody detection. The strain to be tested is combined with high-titre M-type antisera or with appropriate controls. Blood donors of substandard bactericidal effectiveness are not suitable (see Tables 14 and 15).

13.5 Evaluating M-protein content using the indirect bactericidal test

A further use of the indirect bactericidal test is to evaluate strains for their M-protein content. At present it is the most reliable method available for definitively determining the presence of this important epidemiological marker and virulence factor. Although a positive M-type precipitin reaction in gel diffusion using carefully prepared and tested antisera and proper controls can be considered evidence of the presence of M protein, there is always a possibility that an unrelated antigen–antibody reaction could lead to an erroneous conclusion. The extreme specificity for M protein in the properly performed and controlled bactericidal test virtually eliminates this problem.

An important application of the bactericidal test in evaluating M-protein content is in selection of strains for production of M-typing antisera. The prescreening of strains and the selection only of those with optimal growth in human blood will maximize the chances of successful antiserum production.

Strains are frequently erroneously identified in the literature as M negative when they should be more accurately reported as M non-typable. Lack of a

reaction with type-specific anti-M sera may be due to absence of M protein. However, the problem is more likely to be a lack of potent homologous type-specific antiserum. In this situation, the only way to verify that the strain is truly M negative is by means of the indirect bactericidal test.

14. Production of antisera for serological grouping and serotyping

14.1 Grouping antiserum

Accurate clinical diagnosis of a streptococcal infection depends on accurate identification of the serological group of the streptococcal strain isolated. Serological grouping tests have been discussed in detail in section 4. Performance of these tests requires a source of potent anti-group polysaccharide sera; although there are commercial sources available, at times it is advantageous for individual laboratories to prepare their own group-specific antisera. However, laboratories planning to do this should be aware of the many variables that can affect the immune response to streptococcal group antigens. Factors of primary importance include the immunization route and schedule, the physical state of the antigen and host animal genetic factors (*140, 141*). The rabbit has historically been the host animal of choice for this work.

Immunizing strains for group antiserum production can be obtained from the Prague laboratory (the Czechoslovak National Collection of Type Cultures), from other streptococcal research laboratories or from national culture collections such as the American Type Culture Collection in the USA and the National Culture Type Collection in the UK. The vaccine preparation method described below is based on that of Osterland et al. (*142*) and on methods used at the Centers for Disease Control and Prevention in the USA (*143*). An alternative method of vaccine preparation (and immunization) as used in the Prague laboratory is available on request. The bleeding schedule described is from the Centers for Disease Control and Prevention.

Equipment and supplies

— Incubator, 35–37 °C
— Blood agar plates
— Centrifuge
— Sterile membrane filter, 0.2 µm
— Water-baths, 37 °C and 56 °C
— McFarland No. 3 turbidity standard (0.3 ml of 1% (w/v) $BaCl_2$ + 9.7 ml 1% (v/v) H_2SO_4). Alternatively, use a spectrophotometer.
— Rabbits, New Zealand White (or other breed as available), 2.5–3.5 kg
— Syringes, 1 ml, with 25-gauge needles
— Equipment and supplies for bleeding rabbits

Reagents

— Reference streptococcal group strains for immunization
— Todd–Hewitt broth
— Sheep blood
— Pepsin solution (1 mg/ml pepsin in 0.01 ml/l HCl containing 8.5 g/l NaCl); sterilize by filtration
— Saline, 0.85%
— HCl, concentrated and 0.01 mol/l
— NaOH, 0.01 mol/l

Vaccine preparation method (groups A, C and G)

1. Grow the reference group strain overnight in Todd–Hewitt broth containing 5% defibrinated sheep blood.
2. Transfer 2 ml of the overnight culture to a 2-litre flask containing 1 litre of Todd–Hewitt broth.
3. Incubate the 1-litre culture for 18–24 hours at 35–37 °C and streak onto a blood agar plate to test for purity.
4. Centrifuge the overnight culture at 1500 *g* for 30 minutes and resuspend cells in 10 ml of sterile pepsin solution (hydrochloric acid, 0.01 mol/l, in saline containing pepsin, 1 mg/ml; the solution is filter-sterilized).
5. Adjust the pH to 2.0 using concentrated HCl and incubate in a water-bath at 37 °C for 2 hours.
6. Centrifuge, discard the supernatant and resuspend the cells in 10–15 ml of saline.
7. Wash the cells with normal saline and neutralize with sodium hydroxide, 0.01 mol/l. Wash again with saline.
8. Centrifuge as before, resuspend the cells in 10–15 ml of saline and heat-kill at 56 °C for 1 hour in a water-bath.
9. Remove 2 ml of the suspension and prepare a Lancefield acid extract or (preferably) a Fuller extract (see section 4). Test for specificity using reference antisera. Cross-reactions indicate that the vaccine should be discarded.
10. Adjust the vaccine concentration with saline such that a 1:10 dilution is approximately equal to a No. 3 McFarland standard. Alternatively, a 1:20 dilution should have an optical density of approximately 0.4 at 660 nm with a 1-cm path length. If animals die for no apparent reason, dilute the vaccine 1:2.
11. Plate the suspension to test for sterility.
12. Store the vaccine at 4 °C.

Immunization schedule for rabbits

1. Week 1: give three intravenous injections of 0.5 ml of vaccine on successive days.
2. Weeks 2–4: give three 1-ml intravenous injections per week on successive days.
3. Weeks 5: test bleed (small amount) from the ear. If the antibody titre is satisfactory (distinct precipitin reaction with extracts of the immunizing strain as well as several strains of the homologous group, and no or only weak reactions with heterologous reference strains), a large bleed of up to 50 ml may be taken either from the ear or by cardiac puncture. If the titre is not satisfactory, an additional series of three injections can be given and the test bleed repeated. If the rabbit has not responded with satisfactory titres of group-specific antibody after 6 weeks of injections, it should be rested for a minimum of 2 months before reimmunization is attempted.

Bleeding schedule

Large quantities of blood may be obtained by alternating bleeding with booster injections. Bleedings of 50 ml can be repeatedly obtained either by cardiac puncture or from the ear.

1. Week 1: bleed on Wednesday, inject 1 ml of vaccine on Thursday and Friday.
2. Week 2: bleed on Monday and Thursday, inject 1 ml of vaccine on Friday.

3. Continue alternating weeks as long as the titre and specificity of the serum remain satisfactory and the rabbit remains in good health. Typically, 2–4 weeks of this alternating bleeding/immunizing schedule are possible before the quality of the serum begins to decline.

Note: Rabbits that have not responded adequately after the first 6 weeks of injections will frequently give a much better response if they are rested for 2 months or more and then reimmunized. One report indicated that two-thirds of rabbits immunized with group A vaccine showed a significant increase in antibody titre following a second series of immunizations (*140*). The immune response usually occurs much earlier in the second series of injections; the first test bleed should therefore be done after 2 weeks of injections. The second immunization series should begin with three 0.5-ml injections given subcutaneously in the first week followed by three 0.5-ml intravenous injections in the second week. If the serum is not satisfactory at this point, the volume of subsequent intravenous injections should be increased to 1 ml, as used during the initial immunization series.

14.2 T-agglutination antiserum

Preparation of T-agglutination antisera is complicated by the fact that crude antisera always contain nonspecific as well as specific anti-T antibodies, and by the difficulty in discriminating T-dependent from nonspecific agglutination. Production of a monospecific antiserum therefore frequently requires multiple absorptions to remove unwanted antibodies. Also, standardization of antibody titre is of greater importance with T-agglutination antisera than with either group or M-type antisera. The method described below is summarized from Moody et al. (*93*) and is based on methods originally used at the Streptococcus Reference Laboratory, Central Public Health Laboratory, London, England. Streptococcal strains for producing T-agglutination antisera can be obtained from the Prague laboratory as can additional methods for antiserum production. Antisera are also available commercially from several sources.

Equipment and supplies

— Incubator, 30 °C and 35–37 °C
— Blood agar plates
— Centrifuge
— Rabbits, New Zealand White (or other breed as available), 2.5–3.5 kg
— Syringes, 1 ml, with 25-gauge needles
— Equipment and supplies for bleeding rabbits

Reagents

— Reference streptococcal strain(s) for immunization and for testing sera after immunization
— Todd–Hewitt broth
— Trypsin, 1:250–1:300
— Phosphate buffer, 0.01 mol/l, pH 7.8
— Saline, 0.85%
— Formaldehyde, 37%
— Standard T antisera

Vaccine preparation

1. Inoculate a single colony of the immunizing strain into 5 ml of Todd–Hewitt broth and grow for 4 hours at 37 °C.

2. Transfer 1 ml into 220 ml of Todd–Hewitt broth containing 1% trypsin (prepared using trypsin 1:250–300).
3. Incubate overnight at 30 °C.
4. Test the culture for purity by plating on a blood agar plate.
5. Collect the cells by centrifugation at 1500 *g* for 30 minutes and resuspend in phosphate buffer, 0.01 mol/l, pH 7.8, containing 5% trypsin.
6. Incubate overnight at room temperature.
7. Centrifuge as above, wash the cells six times with 0.85% saline and resuspend in 25 ml of saline containing 3% formalin.
8. Test for sterility after 2–3 hours at room temperature. Test for activity using standard T-antisera. Store at 4 °C.
9. Before use, centrifuge as above and replace half of the liquid with fresh saline to reduce the formalin concentration.

Immunization and bleeding schedule for rabbits

1. Week 1: inject one 0.5-ml dose of vaccine intravenously.
2. Weeks 2–5: beginning 5 days after the injection of the previous week, inject 0.5–1.0 ml intravenously on each of three consecutive days.
3. Test bleed 6–10 days after the last injection and test for agglutination activity.
   (*a*) If activity is present, absorb a 1:5 dilution of the serum with "T6 glossy" cells (see below) and test the reaction.
   (*b*) If a good reaction (≥2+) is obtained with a 1:160 serum dilution, bleed 60–80 ml. Bleedings can continue at intervals of 2–7 days as long as the antibody titre is maintained.
   (*c*) If the titre is too low, continue weekly injections and test bleedings for up to 8 weeks.

Absorption of antisera

1. Prepare the absorbing cell suspension.
   (*a*) Inoculate the absorbing strain (see step 9) into 2 litres of Todd-Hewitt broth and grow at 37 °C for 48 hours.
   (*b*) Plate the suspension for purity and heat-kill in a water-bath at 60 °C for 30 minutes.
   (*c*) Allow the suspension to settle overnight at 4 °C. Plate again to check sterility.
   (*d*) Centrifuge at 1500 *g* for 30 minutes and wash three times in 0.85% saline.
   (*e*) Resuspend the cells in 20 ml of phosphate buffer, pH 7.8, and boil for 1 hour.
   (*f*) Centrifuge as above and dispense in volumes containing approximately 1 part packed cells to 4 parts phosphate buffer and store at 4 °C.
2. Prepare the absorbing cells by washing twice with 0.85% saline.
3. Remove the supernatant saline by centrifugation as above and mix 1 volume of cells with 3 volumes of serum.
4. Incubate at room temperature for 30 minutes.
5. Centrifuge as above and decant the serum.
6. Test by slide agglutination using a preparation of the absorbing strain. If agglutination occurs, repeat the absorption.
7. Dilute the absorbed serum with saline and test the homologous reaction. The titre should be ≥160 for satisfactory use.
8. Test a 1:5 dilution of the absorbed serum against all heterologous T types. If heterologous agglutination occurs, an attempt should be made to remove the reaction by absorption with the cross-reacting strain.

9. All antisera are routinely absorbed first with strain T6 glossy (except for T6 antisera, which must be absorbed with another strain). Most antisera give satisfactory results with this absorption alone.
10. Special absorptions may be useful for the following types:
    — types 5, 11, 12, 27 and 44 require special absorption methods as described below;
    — type 3 requires absorption with types 13 and/or B3264;
    — type 9 requires absorption with type 18;
    — type 13 requires absorption with type 3 and/or B3264;
    — type 14 requires absorption with type 49 and vice versa.

Preparation of absorbing antigen for types 5, 11, 12, 27, 44 and types 14 and 49 (refs 144, 145)

1. Grow absorbing strains in 2 litres of 0.5% glucose nutrient broth at 30 °C for 48 hours.
2. Plate the cultures to test for purity.
3. Heat-kill the cells at 80 °C for 10 minutes and allow to stand overnight.
4. Centrifuge at approximately 1500 *g* for 30 minutes, wash twice in 0.85% saline and resuspend in 21.6 ml of phosphate buffer, pH 7.8, and 2.4 ml of 1% trypsin.
5. Adjust the pH to 8.2 using NaOH and heat at 50 °C for 2 hours with mixing every 30 minutes.
6. Centrifuge as above and remove the clear supernatant fluid.
7. Adjust the pH of the supernatant to 2.5 using hydrochloric acid, 1 mol/l, and hold overnight at 4 °C; heavy precipitation occurs. The deposit obtained by centrifugation is the absorbing antigen (T precipitate).
8. Mix 4 ml of serum diluted 1:5 with buffer, pH 7.8 (previously absorbed with T6 glossy), with dry T precipitate from 2 litres of culture.
9. Incubate at 37 °C for 2 hours, then at 4 °C overnight.
10. Centrifuge as above to remove any precipitate and test for cross-reactions by agglutination.
11. The following absorptions are usually carried out:
    — T5 serum with T11 and T12
    — T11 serum with T5 and T12
    — T12 serum with T11 or T27
    — T27 serum with T11 and T12
    — T44 serum with T11 and T12
    — T14 serum with T49
    — T49 serum with T14

Preparation of T-serum pools

Monospecific T antisera should be combined into pools in combinations as described in section 6. The serum dilutions used should be such that the strength of the agglutination reaction obtained with the pool is similar to the strength of the reaction subsequently obtained with the monospecific antiserum. Monospecific antisera are commonly used at a dilution of 1:5 or 1:10.

14.3 M-typing antiserum

Production of M-typing antisera can be among the most difficult tasks in streptococcal microbiology. Some streptococcal strains, especially those that are negative for opacity factor, may be relatively good immunogens and produce good antibody responses in rabbits. However, many produce only

weak responses or no specific responses even after prolonged periods of immunization. Many factors play a role in the success of this endeavour, and a number of papers have been written describing protocols that may improve the anti-M protein response rate. Of prime importance are factors such as the M protein content of the vaccine strain, the method used to prepare the vaccine, and the immunization route and schedule. Methods for enhancing the amount of M protein carried by a strain have been described. These include the classical mouse passage as well as a more recent, and somewhat simpler, method ("blood rotation") mentioned in section 13.1.1, which involves rotating the strain in human blood with or without dilute homologous M-type-specific antibody (*131, 132*). Modifications in vaccine preparations or immunization protocols have also been reported to improve the success of antiserum production (*146–149*). No significant differences in anti-M protein response rates have been observed in different breeds of rabbits (*150*).

Most researchers preparing anti-M protein sera have used a modification of the method developed by Lancefield (*93, 143, 151, 152*). The antiserum production method described below is based on these methods. A number of methods have also been described for the absorption of M-typing antisera (*93, 143, 151, 153, 154*). The absorption methods described at the end of this section are those used by the Prague laboratory.

Equipment and supplies

— Incubator, 35–37 °C
— Blood agar plates
— Centrifuge
— Water-baths, 56 °C and 60 °C
— Steam-bath
— Rabbits, New Zealand White (or other breed as available), 2.5–3.5 kg
— Syringes, 1 ml, with 25-gauge needles
— Equipment and supplies for bleeding rabbits

Reagents

— Reference streptococcal strain(s) for immunization and for testing sera after immunization
— Todd–Hewitt broth
— Saline, 0.85%
— Phosphate buffer, 0.15 mol/l, pH 7.8

Vaccine preparation

1. Select strains for vaccine that have been shown to be M positive by their ability to grow in normal human blood (see section 13).
2. Inoculate 500 ml of Todd–Hewitt broth with a logarithmic growth phase culture of the vaccine strain.
3. Incubate the culture at 35–37 °C overnight.
4. Centrifuge at 1500 *g* for 30 minutes and resuspend cells in approximately 50 ml of 0.85% saline.
5. Plate the suspension to test for purity.
6. Heat-kill the streptococci at 56 °C for 30 minutes.
7. Plate the suspension to test for sterility; if necessary repeat heat inactivation.
8. Remove 5 ml of the suspension and prepare an acid extract. Test the extract for M protein in a precipitin test (if control sera are available).

9. If the vaccine is sterile and has adequate M protein, store at 4 °C until needed.

Immunizing and bleeding schedule for rabbits

1. Week 1: inject 0.5 ml of vaccine intravenously as a sensitizing dose.
2. Weeks 2–4: beginning 5 days after the sensitizing dose, inject 1.0 ml intravenously on each of three consecutive days.
3 Week 5: test bleed 5 days after the last injection. Collect serum and test for precipitin activity. If this is done in capillary tubes, refined extracts from which group substance has been removed must be used (*93*). If the Ouchterlony double-diffusion test is employed, standard Lancefield extracts can be used (see section 7).
4. If the response is satisfactory, the rabbit can be bled following the schedule given in section 14.1 for production of grouping antiserum.
5. If the response is not satisfactory, immunizations should continue following the schedule for weeks 2–4. Test bleed each week. If a satisfactory response is not obtained after 7 weeks, the immunizations should be stopped and the rabbit reimmunized after a rest of 2–3 months. Subsequent immunization should follow the schedule given for production of grouping antiserum (see section 14.1).
6. If the Ouchterlony double-diffusion test is used for typing, each crude antiserum that reacts with the homologous extract must be checked for reactions with M extracts of all available types as well as with group A polysaccharide. Cross-reacting antisera require absorption. For the capillary precipitin test only absorbed antisera can be used.

Absorption of M-typing antisera

Method A

1. Inoculate the strain used for absorption into 2 litres of Todd–Hewitt broth and incubate at 37 °C for 48 hours. Strains T6 glossy and "T25 Matthews" are routinely used for the first absorption.
2. Check the strain for purity, heat at 60 °C for 30 minutes and centrifuge at 1500 *g* for 30 minutes or leave overnight to settle.
3. Draw off the supernatant and wash the cells three times in saline.
4. Resuspend the cells in one-tenth of the original volume of phosphate buffer (0.15 mol/l), pH 7.8.
5. Heat at 100 °C for 1 hour and store at 4 °C as the stock suspension.
6. Wash the desired volume of the stock suspension from step 5 twice in saline.
7. Pack the cells tightly by centrifuging as above and draw off the supernatant.
8. To one volume of cells add three volumes of antiserum.
9. Shake well and leave at room temperature for 30 minutes.
10. Centrifuge as above to remove the cells and test the antiserum either in capillaries or using the double-diffusion test with extracts of all available types. Some antisera need further absorption with other heterologous strains.

Method B

If the double-diffusion test is used for typing, the following method for absorption of sera, based on a procedure described by Schmidt (*155*), can be used to great advantage.

1. Dissolve a freeze-dried hydrochloric acid extract of the heterologous (cross-reaction) strain with one-half of its original volume of the antiserum to be absorbed.
2. Types M58, M49, M25, M9 or M2 are usually used because they yield the most frequent cross-reactions.
3. Incubate this mixture for 2 hours at 37 °C and then overnight at 4 °C.
4. Centrifuge at 1500*g* for 30 minutes to remove any precipitate and test (double-diffusion) with all available hydrochloric acid extracts.

14.4 Opacity-factor typing antiserum

As indicated in section 8, inhibition of the serum opacity reaction has become a valuable tool for the streptococcal epidemiologist. A number of researchers have found that human sera frequently contain antibodies to streptococcal opacity factor and that these antibodies can be used satisfactorily in serotyping tests (*100, 108–111, 156*). The primary disadvantage of the use of human serum is that multiple antibodies are nearly always present. Therefore, it is *never* possible to rely on inhibition by a single human serum to identify the opacity-factor type of an unknown strain. Use of monospecific hyperimmune animal sera is preferred whenever possible, and a number of methods have been described for producing these sera (*100, 104, 112, 157, 158*). Although a number of researchers have used rabbits for the production of opacity factor antibodies, improved success has been reported using guinea-pigs (*157*). This method is described below.

Vaccine preparation

1. Prepare enriched Todd–Hewitt broth.
   (*a*) Supplement with an additional 2% (w/v) neopeptone.
   (*b*) Buffer with the addition of disodium hydrogen phosphate dihydrate, 0.74 g/l of Todd–Hewitt broth, and sodium dihydrogen phosphate dihydrate, 0.13 g/l of Todd–Hewitt broth.
   (*c*) Adjust the pH to 7.4 and sterilize by autoclaving for 5 minutes at 115 °C.
2. Inoculate 250 ml of enriched Todd–Hewitt broth with 5 ml of logarithmic growth phase culture of vaccine strain and grow for 18 hours at 35–37 °C.
3. Centrifuge at 1500*g* for 30 minutes and wash the cells three times with 0.85% saline.
4. Resuspend the cells in 25 ml of phosphate buffer, 0.1 mol/l, pH 7.8.
5. Remove 5 ml for preparation of a Lancefield acid extract (see section 7).
6. Plate the suspension to test for purity.
7. Heat-kill the vaccine at 56 °C for 30 minutes. Cool and plate again to test for sterility. If necessary, repeat heat inactivation.
8. Test the vaccine for opacity factor content by spotting a drop of the Lancefield extract on a horse or pig serum agar plate or by testing by the microtitre plate method (see sections 8.3 and 8.6).

Immunization and bleeding schedule

1. Use white Dunkin–Hartley guinea-pigs, 450–500 g in weight.
2. Week 1: inject 0.25 ml of vaccine subcutaneously under the loose skin at the back of the neck.
3. Week 2: 5 days after the sensitizing dose, inject 0.25 ml subcutaneously and 0.5 ml intraperitoneally.
4. Weeks 3–5: inject 0.25 ml subcutaneously and 0.5 ml intraperitoneally once weekly.

5. Week 6: test bleed. If the response is unsatisfactory, the immunization schedule given for weeks 3–5 can be continued for up to 8 weeks.
6. If no response occurs after 8 weeks, rest the animal for 2 weeks and then reimmunize for an additional 4 weeks following the schedule given in step 4 for weeks 3–5.
7. Check antisera that react with homologous antigen (supernatant, extract) for specificity with all available opacity factor types. Discard cross-reacting antisera (these occur only rarely). High-titre anti-opacity factor sera may satisfactorily be used diluted with 0.85% saline.

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