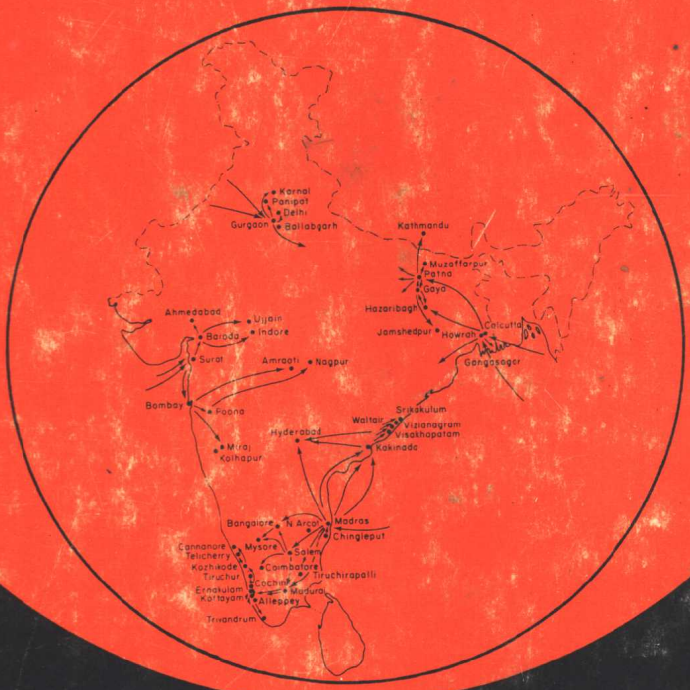
METHODS IN MICROBIOLOGY



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PREFACE

Volume 12 of "Methods in Microbiology" continues the series of four Volumes which describe the methods available for typing the major human pathogens.

Volume 12 begins with a general article on the serotyping of bacteria and then presents three short Chapters on the characterisation of *Yersinia enterocolitica*. Two Chapters deal with *Vibrio cholerae* and the rest of the Volume is concerned with the Gram-positive cocci including Chapters on *Staphylococcus*, haemolytic streptococci, enterococci, bacteriocin typing of streptococci and the identification and characterisation of *Streptococcus pneumoniae*.

As with Volumes 10 and 11, we consider ourselves fortunate in having recruited internationally recognized authorities on these various topics as authors. We believe that the resulting compendium of methods, together with the detailed discussion of the epidemiological backgrounds, will provide a valuable reference work for microbiologists working in the epidemiological field and a valuable orientation for newcomers to this important area of microbiology.

T. BERGAN
J. R. NORRIS

October, 1978

CONTENTS

CHAPTER I

Serotyping of Bacteria

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I. INTRODUCTION

The use of serological methods for epidemiological typing is based upon the fact that micro-organisms frequently show variations in antigenic constitution, not only between distantly related or unrelated organisms, but even within groups of closely related organisms. The identification of the antigens of micro-organisms by means of specific antibodies, therefore, often allows very fine subdivision of microbial taxa, and greatly facilitates the surveillance and control of the spread of pathogenic micro-organisms.

Micro-organisms produce a wide variety of antigens, many of which can be utilised in identification and epidemiological typing: structural components of the cells, such as cell wall constituents, capsules or envelopes, flagellae, fimbriae; secretion products of the cells such as exotoxins or

extracellular enzymes; or antigens contained in the interior of the cells. Many of these antigens can be utilised for typing purposes.

Chemically the antigens used for such purposes are of two main kinds: proteins and carbohydrates (including compounds composed of both polypeptide and carbohydrate components). Antigens are generally large molecules of complex structure, and, therefore, they frequently have more than one substructure which can serve as an antigenic determinant. Immune sera against antigens accordingly may contain separate antibodies against the various antigenic determinants on the same molecule, and even antibodies reacting with the same determinant may be heterogeneous molecules with different specificities, i.e. different degrees of complementarity to the same determinant.

II. SPECIFICITY

The basis of the use of serological reactions is their specificity, the fact that antibodies produced in response to immunisation with one particular antigen give maximal reaction with that antigen only, i.e. a substance, or a substructure (a determinant) on an antigen with identical chemical constitution.

III. CROSS-REACTIONS AND THEIR SIGNIFICANCE

In the early stages of the development of immunology the specificity of serological reactions was thought to be nearly absolute, with only few known exceptions. But accumulated experience has shown that this is not so. Serological cross-reactions are known to be of common occurrence and must be taken into consideration in the evaluation of the results of serological tests. With respect to cross-reactions there is a principal difference between protein antigens (i.e. antigens where the determinants are of protein nature) and carbohydrate antigens (antigens where the determinants are of carbohydrate nature).

In the case of carbohydrate antigens, cross-reactions are quite common and often occur between antigens from entirely unrelated organisms. Such cross-reactions are due to structural similarities, which may be quite accidental, such as shared monosaccharides in similar linkages. The principles of such cross-reactions have been eminently elucidated by Heidelberger and his co-workers in a large number of studies, and by other authors. As a consequence of such studies it is clear that cross-reactions between polysaccharide antigens only indicate structural similarity and do not necessarily indicate genetic relationship of the organisms producing the antigens.

With protein antigens the situation is quite different. As a general rule protein antigens do not cross-react with other proteins. But there is one very important exception: homologous, iso-functional proteins (i.e. of common phylogenetic origin and with identical function, e.g. enzymes) from different organisms may show cross-reactions (Cocks and Wilson, 1972; Gordon *et al.*, 1969; Holten, 1974; Murphy and Mills, 1969; Steffen *et al.*, 1973) which may vary in strength according to the degree of phylogenetic relationship between the organisms and to the rate of evolution of the protein in question, i.e. the frequency of mutational changes of the amino-acid sequence. Such cross-reactions can be utilised in measuring phylogenetic relationships between organisms. Cross-reactions between proteins due to accidental structural similarity appear to be unknown.

IV. GENETIC DETERMINATION OF ANTIGENS

Many antigens are genetically determined by structural genes in the genome of the organism and are reasonably constant. But some antigens are determined by other, extraneous genetic elements, e.g. bacteriophages or by plasmids or episomes. Thus protein antigens such as exotoxins (Barksdale *et al.*, 1961; Zabriskie, 1964) and O-antigen factors in Gram-negative bacteria (Iseki and Kashiwagi, 1955; Iseki and Sakai, 1954; Le Minor, 1963a, b, 1965a, b, c, 1966a, b, 1968; Le Minor *et al.*, 1963; Le Minor *et al.*, 1961; Staub and Forest, 1963) may appear or disappear with the entry or exit of a prophage from the genome. Antigens may also be modified by mutation, e.g. the S–R dissociation in enteric Gram-negative rods, which alters the O-antigen by eliminating side chains from its "core" polysaccharide (Kauffmann, 1961).

V. DEMONSTRATION OF ANTIGENS

Antigens with only a single known determinant, eliciting antibodies of only one specificity, e.g. *Salmonella* O-antigens 11 or 16, can be demonstrated by the use of an untreated immune serum from a rabbit. But when the antigen is a mosaic of several determinants, each eliciting production of a separate antibody, it may be necessary to prepare factor sera, reacting only with one determinant, by absorbing the serum with organisms which possess one or more, but not all, of the same determinants. The antibodies which react with the determinants on the absorbing antigen are thus removed, whereas the antibodies which fail to react with any determinant on the absorbing antigen, remain in the serum. For example antiserum against *Salmonella paratyphi A* O-antigen (O: 1, 2, 12) contains antibodies

anti-1, anti-2 and anti-12. By absorption with the *durazzo* variety of *S. paratyphi A* (O: 2, 12) the two latter antibodies are removed and only anti-1 remains. Many other examples may be found in Kauffmann (1961).

VI. INTERPRETATION OF SEROLOGICAL REACTIONS

When an unknown microbial antigen reacts with an immune serum of known specificity, this may in principle be explained in two different ways: (a) either the unknown antigen may be identical with the one used to raise the immune serum, in which case titres of the serological reactions usually will be equal with the unknown and the homologous antigens, or (b) it may be a cross-reaction, in which case the titres given by the unknown antigen may be the same as, or lower than with the homologous antigen. In order to choose between the two alternatives, it may be necessary to carry out absorption of the serum with the unknown antigen. If this leads to complete exhaustion of the serum of all antibodies reacting with the homologous and the unknown antigens, the two antigens can be considered identical. But if only the antibody reacting with the unknown antigen is removed, whereas some of the antibody reacting with the homologous antigen remains, the two antigens are not identical.

In the evaluation of serological reactions it is important to realize that cross-reactions can be of two kinds: They may be due to sharing by the two antigens of one or more, but not all, identical antigenic determinants (e.g. O-antigens 1, 4, 5, 12 and 1, 9, 12 in the *Salmonella* group), or to similar, but not identical chemical structure of one or more determinants on the two antigens (e.g. the capsular polysaccharides of types 3 and 8 of *Streptococcus pneumoniae*, the former being a polymer of cellobiuronic acid, and the latter containing cellobiuronic acid units separated by one glucose and one galactose residue). Cross-reactions can be of all degrees, and they can be bilateral or unilateral.

VII. KINDS OF SEROLOGICAL REACTIONS

A large variety of serological reactions are available. The reactions most commonly used for epidemiological typing are briefly presented in the following.

A. Simple reactions requiring only antigen and antibody

1. *The precipitation reaction*

This is a reaction between antigen in a colloidal solution and antibody, where lattices are formed and give visible precipitation. The lattice formation is due to the fact that each antibody molecule has two or more

antigen binding sites, "valencies", and the antigen molecules, likewise, have several determinants to which antibody molecules can be bound and therefore the antigen functions as a multivalent substance. Variations in the relative proportions of antigen to antibody cause continuous variation in the composition of the precipitate. In the region of antibody excess the precipitate tends to be white, compact, dehydrated and insoluble. Towards the zone of antigen excess the precipitate becomes more translucent and gelatinous, more highly hydrated and increasingly soluble, until, in extreme antigen excess only soluble antigen-antibody complexes are formed. The composition of the precipitate is to some extent dependent upon the ionic strength of the solution.

The reaction exists in several modifications:

(a) *The simple ring test.* The antigen solution (often in ten-fold dilutions) is layered over immune serum in narrow tubes or capillaries. In positive reactions a disc of precipitate forms at the interface. The reaction is useful for qualitative demonstration or semi-quantitative measurement of antigens. Grouping and typing of streptococci is an example.

(b) *The quantitative precipitin reaction,* where precipitates formed by known volumes of antigen and serum are washed and the nitrogen content measured chemically. The reaction is not much used for typing purposes, but finds wide application as a research tool.

(c) *The capsular swelling reaction.* Dilute suspensions of encapsulated organisms, mostly bacteria, are mixed with serial dilutions of specific immune serum on slides. In positive cases antibody is bound to the outer layers of the capsules, which then become visible in the microscope, giving the impression that the capsule has swelled. The titre, the highest serum dilution giving a positive reaction, may be useful in distinguishing between a homologous reaction and a cross-reaction. The reaction is used for example, in typing of *Klebsiella* and *Haemophilus.*

(d) *Gel diffusion methods.* In single diffusion an antigen solution is layered over an agar column containing immune serum in a tube or capillary. When antigens and corresponding antibodies after diffusion meet in optimal proportions in the gel, precipitate bands appear, one or more according to the number of antigen–antibody systems involved. This reaction also finds little general application in typing. Double diffusion in tubes may similarly be set up: a layer of immune serum in the bottom, then a layer of clear agar and on top a layer of antigen solution. After diffusion precipitate bands form in the agar layer.

In double diffusion in agar layers, holes ("wells") are made in an agar layer in a Petri dish or on a glass plate. The number and arrangement of the

wells can be varied at will, but the distances between the wells must always be such that visible precipitates can be formed between antigen wells and antiserum wells. Some wells are filled with antigen solutions, others with serum, or antibody solutions, and the dishes are incubated in a humid atmosphere until precipitates have appeared. Again one or more precipitate bands may form between an antigen well and a serum well according to the number of antigen–antibody systems. The method permits comparison of antigens against the same antibody (or vice versa), and it is possible to distinguish reactions of identity, cross-reactions or reactions of non-identity. A technique using cellulose acetate membranes instead of agar layers has also been developed. This method can be applied to many different problems, for example typing of capsular antigens or of viruses.

(e) More sophisticated methods are also available, and could be utilised for typing purposes: immunoelectrophoresis in agar cells or on cellulose acetate membranes, radio-immunoelectrophoresis, two-dimensional electrophoresis. A description of such methods would be outside the scope of this Chapter.

2. *The agglutination reaction*

This is a reaction between an antigen in the form of a suspension of particles and antibody. In positive reactions, the particles are bound together by antibody molecules with two or more antigen binding sites. The reaction may be carried out as a direct agglutination, where the antigen is a suspension of the organism in question: bacteria, *Rickettsia*, fungal elements. Such reactions may be carried out as qualitative tests, usually on slides, by suspending organisms from a culture on solid medium in a drop of serum, or serum dilution. Tests for demonstration of bacterial antigens such as *Salmonella* H- or O-antigens are usually carried out in this manner. The sera must have been carefully checked beforehand for reactivity and specificity, and for determination for the most suitable test dilution.

Agglutination may also be carried out in test-tubes by mixing serial (mostly two-fold) dilutions of serum with equal volumes of antigen. According to the nature of the antigen, incubation time and temperature may be varied. Agglutination of bacterial H-antigens by the corresponding antibody proceeds comparatively rapidly and is usually completed in 2–4 h. O-agglutination is slower and requires incubation overnight to be completed. The tests may be incubated in a water bath at 37 or 50 °C.

Agglutination reactions may also be carried out as indirect or "passive" agglutination, where the antigen, usually extracted from an organism, is

bound to an inactive particle, for example latex or bentonite, or to erythrocytes (indirect haemagglutination). Antigen may be bound to untreated erythrocytes (lipopolysaccharides), to tanned corpuscles (proteins) or be fixed to the corpuscles by means of chromium chloride (certain polysaccharides). Before carrying out the test it may be necessary to absorb the serum with untreated erythrocytes to remove possible natural antibodies.

Indirect agglutination reactions are usually more sensitive than precipitation reactions with the same antigens. The indirect haemagglutination reaction in particular has been applied to numerous problems, including demonstration of bacterial antigens.

The titrations can be carried out by the conventional technique in tubes, or on plastic trays by the microtitre technique introduced by Takatsy (1955).

B. Reactions requiring one or more additional components, e.g. complement or indicator systems

1. *Cytolysis–bacteriolysis*

When certain cells such as erythrocytes or bacteria e.g. *Vibrio cholerae* or some other Gram-negative rods, are exposed to specific antibodies and complement, the cell membranes are damaged and the cells lysed (the lysis of *Leptospira* cells by antibody is complement independent and does not belong in this category). If erythrocytes, coated with antigens on their surface are exposed to antibodies against the coating antigen and complement, lysis may also occur. Such reactions might be applied to typing, but apparently are not much used for such purposes at the present time.

2. *Complement fixation*

The formation of antigen-antibody complexes in the presence of complement causes activation of complement. One of the consequences of complement activation is the disappearance of its haemolytic activity. This is called "complement fixation". The loss of haemolytic activity may, therefore, be taken as evidence that an antigen–antibody reaction has occurred.

The reaction is carried out in two steps. In the first step a mixture of an antigen (known or unknown), a serum (unknown or known respectively) and complement is incubated in order that the antigen-antibody reaction and subsequent complement activation may take place. In the second step a test for remaining haemolytic complement activity is carried out by adding "sensitised" sheep erythrocytes (erythrocytes "coated" by rabbit antibody against sheep erythrocytes, immune haemolysin or "ambo-

ceptor"). If haemolysis occurs, it means that haemolytic complement still is available, complement has not been activated, the reaction is negative. Absence of haemolysis indicates that activation of complement has taken place, which, provided that all controls are satisfactory, indicates an antigen–antibody reaction, i.e. the reaction is positive. The reaction can be carried out by the conventional test-tube titration, or as a micro-modification in plastic trays with microtitre equipment according to Takatsy (1955).

Many different modifications of the test have been used, and it would be outside the scope of this Chapter to describe them. Only a few comments on the principles of the test are given here.

(a) *Complement.* The usual source of complement is fresh guinea pig serum. Guinea pig blood is drawn by heart puncture. As soon as possible after coagulation and clot retraction, the serum is separated from the clot and centrifuged. If it is to be used within 24 h, it may be stored at *c.* 4 °C. If not, it may be maintained in an active state by storage below −20 °C or by lyophilisation. When complement is being used to set up tests, it should preferably be kept cold in ice water to avoid inactivation at high room temperature.

In crude routine tests 0·85% saline is often used as diluent, but due to the fact that two of the steps in the complement activation require the presence of calcium ions and magnesium ions, respectively, saline does not ensure optimal complement activity, as the concentrations of these ions may be too low. It is preferable, therefore, to use a barbiturate buffer, as described by Mayer (1961), or a tris buffer as described by Levine (1967), which contain optimal amounts of these ions.

Because complement is a very unstable substance, a titration of its activity should be carried out on each occasion when it is going to be used. This is done by testing varying quantities of complement against constant quantities of immune haemolysin and of sheep erythrocytes. If the degree of haemolysis is plotted against the quantity of complement, a sigmoid curve is obtained, with the steepest slope corresponding to about 50% haemolysis. The most exact way to measure complement activity, therefore, is to determine the dilution that produces exactly 50% haemolysis. Determination of the dilution that produces 100% haemolysis (which is often done), is much less accurate. In a complement fixation test it is necessary to use a slight excess of complement, as some of the other reagents used may have a slight anticomplementary effect. It is customary, therefore, to use an arbitrary number of 50% haemolytic units per unit volume, e.g. 4, 50% units. In complement fixation tests one must always include a complement control. i.e. a control with complement alone, without serum

or antigen, to make sure that complement activity has been maintained during the test period.

(b) *Serum.* In complement fixation tests for typing purposes the serum will generally be an immune serum of known specificity. In order to make sure that the serum does not contain any complement, it is always inactivated, heated in a water bath at 56 °C for 30 min, before use. Serial dilutions, often two-fold, are usually tested against constant quantities of antigen and complement. A serum control, a tube with serum and complement, but without antigen, should always be set up to make sure that the serum does not contain anticomplementary substances.

(c) *Immune haemolysin.* This is usually raised in rabbits by giving them several injections of suspensions of washed sheep erythrocytes intravenously, e.g. twice a week for 3 or 4 weeks. IgM antibodies, which appear early in the course of immunisation, are more active in immune haemolysis than the later appearing IgG antibodies, and it is therefore preferable not to continue the immunisation longer than necessary to get a good haemolytic titre. The serum is inactivated before use to get rid of any complement it may contain. The serum may be preserved with merthiolate 0·01% or sodium azide 0·1%, and will keep for a very long time at 4 °C, or it may be stored frozen below −20 °C. Each new serum has to be titrated to determine the titre and dilution to be used in complement fixation tests. The titration is carried out by testing twofold dilutions (usually starting at 1:100) of the serum against constant quantities of sheep erythrocytes and complement. As with complement, a moderate excess of haemolysin is also needed in the complement fixation reaction, e.g. two to four times the concentration needed to produce complete haemolysis in the titration.

(d) *Antigen.* A good antigen should have a high capacity for reacting with specific antibody and it should not be anticomplementary by itself. For this reason antigen to be used in a complement fixation test should be titrated against constant quantities of complement, but without antibodies, in order to determine whether it is anticomplementary or not. If it has an anticomplementary effect in higher concentration, it is important to select a dilution well below the anticomplementary level, e.g. not more than 1/4 of the lowest anticomplementary concentration. In the actual test a control with antigen and complement, but without serum should always be included.

(e) *Sheep erythrocytes.* Sheep blood is drawn into a sterile modified Alsever's solution (Mayer, 1961) and stored in the refrigerator at *c.* 4 °C. It is recommended that the blood should be left in the refrigerator for about

1 week before use, for stabilisation of its susceptibility to haemolysis. After the lapse of this period of time, the erythrocytes can be used for about 2 months.

Before use a suitable quantity of blood is centrifuged and plasma and buffy coat are pipetted off. The cells are then washed three times in 5–10 volumes of the isotonic diluent (Mayer, 1961). After the final centrifugation a suspension may be prepared by suspending 2–4% packed cells in saline (v/v), or, in more critical work, the cell suspension may be standardised spectrophotometrically to a selected optical density.

Complement fixation tests can be carried out by testing serial (usually twofold) serum dilutions against constant quantities of antigen and complement, or it may be carried out as checker board titrations by testing serial dilutions of serum against serial dilutions of antigen, and with constant complement.

5. *Neutralisation tests, requiring an indicator system*

The indicator system may be experimental animals as in exotoxin neutralisation tests (e.g. typing of toxins of *Clostridium botulinum*), cell cultures (e.g. virus neutralisation), leucocytes (opsonin tests), guinea pigs (passive cutaneous anaphylaxis). Not all these tests are at present being used for epidemiological typing, but they are potentially useful.

C. Reactions using labelled reagents

By the use of fluorescent antibodies (labelled with substances like Fluorescein or Rhodamine-lissamine) it is possible to demonstrate microbial antigens. Antibodies labelled with ferritin or with peroxidase can be utilised for demonstrating microbial antigens by electron microscopy.

VIII. CONTROLS

In order to get reliable and reproducible results of serological tests, it is essential that controls are included in the tests: controls with known antigens in tests designed to demonstrate antigens in unknown specimens, controls of the activity of immune sera, controls of all reagents which are subject to variation: erythrocytes, complement, etc.

IX. PREPARATION AND HANDLING OF IMMUNE SERA

So many different methods have been used for the production of immune sera, that it is impossible to suggest a single method. Many methods and schedules of immunisation have been successful.

In general the rabbit is the experimental animal of choice, unless special reasons speak in favour of using a different animal.

Some antigens, e.g. bacterial suspensions, are very effective and simple intravenous injections in the ear veins give good results. Schedules such as two or three injections per week for 3 or 4 weeks are usually successful. Test bleedings from the ear may be taken 4 or 5 days after the last injection, and the animal may be bled from the heart the following day if the test bleeding proves satisfactory. If not, additional injections may be given until satisfactory titres of antibodies are obtained. It is important to select antigen doses that are non-toxic to the animals, otherwise it is impossible to recommend any particular dosage.

With some antigens, e.g. some protein antigens, it is less easy to produce potent immune sera. In these cases adjuvants may be used. The antigen may, for example, be emulsified in Freund's adjuvant (commercially available). Various schedules have been successful. In some cases a single dose, divided between four or five intramuscular sites is sufficient for good antibody production. In other cases the intramuscular injection of antigen in complete Freund's adjuvant may be followed about 4 weeks later by a new injection of antigen in incomplete Freund's adjuvant, or by an intravenous injection. Additional intravenous injections may be given at intervals of a few weeks.

Blood from immunised rabbits is drawn by heart puncture. The serum, after separation from the clot and centrifugation may be preserved with merthiolate (0·01%) or sodium azide (0·1%). Immune sera may be stored in the refrigerator at *c.* 4 °C, and will keep for a very long time (often for a large number of years) if bacterial or fungal growth is avoided. Sera should always be handled using aseptic techniques. Sera may also be preserved by freezing below −20 °C or by lyophilisation.

X. IMPORTANCE OF THE NATURE OF THE ANTIBODIES

The nature of the antibodies is of some importance in serological tests. Antibodies of the IgM class are much more active than IgG, give higher titres, in certain reactions such as the agglutination reactions or in immune haemolysis. The specificity of the antibodies also appears to vary with the antibody class. Thus experience has indicated that antibodies of the IgM class, e.g. in horse sera against pneumococcus polysaccharides, have a broader specificity, giving more cross-reactions than antibodies of IgG, e.g. rabbit antisera against the same antigens.

XI. SENSITIVITY OF SEROLOGICAL REACTIONS

An important consideration in the selection of serological reactions for