METHODS FOR THE DETERMINATION OF PNEUMOCOCCUS TYPES.*

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It has been shown by Cole[1] and his associates that 75 to 80 per cent of cases of lobar pneumonia in adults are caused by three fixed types of highly parasitic pneumococci which can be differentiated readily by immunological methods, and that the remaining 20 to 25 per cent of cases are caused by a heterogeneous group of serologically independent varieties of pneumococci. These types have been classified by Dochez and Gillespie[2] primarily by means of animal protection experiments into Groups I, II, III (*Pneumococcus mucosus*), and IV (heterogeneous group). It has been shown that classification by agglutination corresponds to the classification by animal protection. Avery[3] has further shown that there is a considerable group of pneumococci which are related to Group II organisms in that mice are protected against infection with such atypical Group II pneumococci by Type II antipneumococcus immune serum and in that such pneumococci are agglutinable by Type II immune serum, though less rapidly and completely than Type II pneumococci. Immune sera prepared by the immunization of animals against such atypical Group II pneumococci, however, fail to agglutinate Type II organisms or to protect mice against infection with Type II strains. These atypical Group II pneumococci have been classified as Subgroups IIa, IIb, and IIx (heterogeneous). No such atypical strains have been encountered in Groups I or III. Statistical studies have shown that the mortality rate in pneumonia varies with the type of pneumococcus causing the disease, the percentage of deaths in each group being fairly constant from year to year. In a series of 400 cases[4] not receiving specific serum treatment the mortality rate was as follows: Group I, 25.2 per cent; Group II, 28 per cent; Group III, 56 per cent; Group IV, 14 per cent. These facts have be-

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[1] Cole, R., *Arch. Int. Med.*, 1914, xiv, 56; *N. Y. Med. J.*, 1915, ci, 1, 58; *Tr. Cong. Am. Phys. and Surg.*, 1916, x, 138.

[2] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

[3] Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

[4] Moore, H. F., and Chesney, A. M., *Arch. Int. Med.*, 1917, xix, 611.

come a valuable guide in prognosis and have provided a basis for rational specific serum therapy in lobar pneumonia which at present has been successfully developed for cases of pneumonia caused by Type I pneumococci.

The determination of pneumococcus types in cases of lobar pneumonia is rapidly coming into extended use because of its value as a prerequisite for specific serum treatment and in the field of prognosis. It is essential that a reliable and standard technique for the determination of types should be used and that the method should be as rapid as possible in order that serum treatment, when indicated, may be instituted at the earliest possible moment.

The method in common use consists in the intraperitoneal injection into a mouse of a specimen of the patient's sputum. By this means a rapid growth of the pneumococcus is obtained while other secondary organisms are usually inhibited. After a suitable interval, varying in the individual case from 5 to 24 hours as determined by preliminary peritoneal puncture with a capillary pipette, the mouse is killed and the peritoneal exudate is washed out with 4 to 5 cc. of normal salt solution. The leukocytes and fibrin are removed from the peritoneal washings by centrifugalization at low speed; the supernatant bacterial suspension is decanted into a second centrifuge tube and whirled at high speed until the bacteria are thrown down. This supernatant fluid is discarded, and the bacterial sediment resuspended in normal salt solution. The type of pneumococcus present is then determined by macroscopic agglutination tests with undiluted antipneumococcus immune serum. At the time of mouse autopsy cultures of the peritoneal exudate and heart's blood are made in broth and on blood agar plates for subsequent confirmation of the determination of type made on the peritoneal washings.

Certain factors have interfered with the rapid determination of types by this method in an appreciable number of cases. The most frequent difficulty encountered has been the growth of secondary organisms in the peritoneal exudate together with the pneumococcus, notably *Bacillus influenzæ* and less frequently *Micrococcus catarrhalis*, staphylococci, streptococci, and *Bacillus typhi murium*. When this occurs agglutination of the pneumococci present is either inhibited or markedly delayed, or spontaneous agglutination occasionally occurs in all tubes. A delay of 18 to 24 hours results until ag-

glutination tests can be made on pure cultures of the pneumococcus obtained from cultures made of the heart's blood at the time of mouse autopsy. When *Bacillus influenzæ* or *Bacillus typhi murium* are present in the peritoneal exudate they are usually present also in the heart's blood cultures and further delay takes place through the necessity of fishing and culturing pneumococcus colonies from the blood agar plate.

A further difficulty which has been encountered recently with the agglutination technique commonly used has been the occurrence of a small number of strains of pneumococci properly belonging in Group IV which agglutinate in all three types of antipneumococcus serum. The character of the agglutination is less rapid and less complete than that which occurs with the fixed types of pneumococci, resembling the agglutination of Subgroup II pneumococci by Type II serum. This phenomenon of cross agglutination is one that might be expected from our knowledge of other bacterial groups and it in no way invalidates the classification of the parasitic pneumococci into sharply defined immunological groups. It rather indicates that with the use of undiluted serum of high agglutinin titer a zone of non-specific reaction is encountered. While only a relatively small number of pneumococcus strains exhibit the phenomenon of cross agglutination, it has been shown by the more delicate precipitin test that a limited zone of non-specific reaction exists for strains of pneumococci of all four groups. It is necessary to give but one protocol of a considerable series to demonstrate this (Table I).

TABLE I.

Determination of the Zone of Non-Specific Precipitin Reaction, Using Pneumococcus Type II Antigen and Antipneumococcus Immune Sera I, II, and III.

Type II antigen 0.5 cc.	1:100	1:500	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000	1:300,000	1:500,000
Serum I (1:10) 0.5 cc.	++	±+	+	±	−	−	−	−	−
" II (1:10) 0.5 "	+++	+++	+++	+++	+++	++	+±	±	−
" III (1:10) 0.5 "	++	++	+±	+	±	−	−	−	−
Normal horse serum (1:10) 0.5 cc.	−								

The reactions were read after 2 hours at 37°C. and over night on ice. Pneumococcus II antigen was prepared by drying *in vacuo* the washed pneumococci from an 18 hour 1,000 cc. bouillon culture. The dried bacterial bodies were taken up in salt solution (10 mg. per cubic centimeter) and the suspension was repeatedly frozen and thawed until a faintly opalescent fluid free from bacterial bodies was obtained. Dilutions of the antigen were made in 0.85 per cent salt solution.

From Table I it is evident that a zone of non-specific precipitation occurs with Pneumococcus II antigen in dilutions not greater than 1:10,000 and that when higher dilutions are used the reaction becomes specific. Similar results have been obtained with antigen prepared from strains of Pneumococcus Type I and Pneumococcus Type III. Group IV pneumococcus antigens show a precipitin reaction only within the limits of the non-specific zone.

To obviate these difficulties certain improvements in the technique for the determination of pneumococcus types have been developed and will be presented below. The preliminary steps are the same as in the commonly used method and will be given in detail for the sake of completeness.

Inoculation of Mice with Sputum.

Collection of Sputum.—Care should be exercised in the collection of sputum to obtain a specimen from the deeper air passages as free as possible from saliva. This can be done in practically all cases, even the most difficult, with a little persistence. The physician or nurse should personally superintend the collection of sputum inducing the patient to cough until a suitable specimen is raised, care being taken not to allow the patient to swallow the lung sputum. The sputum is collected in a sterile Esmarch dish or other suitable container and should be sent at once to the laboratory for mouse injection. When delay is unavoidable the specimen should be kept on ice during the interval.

Microscopic Examination of the Sputum.—Direct films are made from the sputum and stained by Gram's method, with 10 per cent aqueous saffranin as a counterstain, by Ziehl-Neelson's stain, and

by Hiss's capsule stain. This serves to give an idea of the nature of the organisms present and an indication of the source of the sputum. Suitable lung specimens are relatively free, in most instances, from contaminating mouth organisms. It is frequently possible to identify Type III (*Pneumococcus mucosus*) organisms when they are present, as they possess a very large distinct capsule staining by both Gram's and Hiss's methods.

Mouse Inoculation.—A small portion of the sputum, about the size of a bean, is selected and washed through three or four changes of sterile salt solution in sterile Esmarch or Petri dishes to remove surface contaminations. The washed sputum is then transferred to a sterile mortar, ground up, and emulsified with about 1 cc. of sterile bouillon or salt solution, added drop by drop, until a homogeneous emulsion is obtained that will readily pass through the needle of a small syringe. 0.5 to 1 cc. of this emulsion is inoculated intraperitoneally into a white mouse with a sterile syringe. The pneumococcus grows rapidly in the mouse peritoneum while the majority of saprophytic mouth organisms rapidly die off with the exceptions noted above, *Bacillus influenzæ*, and occasionally *Micrococcus catarrhalis*, staphylococci, and streptococci. Pneumococcal invasion of the blood stream also occurs early. *Bacillus influenzæ* like wise invades the blood stream if present; other organisms as a rule do not.

The time elapsing before there is a sufficient growth of the pneumococcus in the mouse peritoneum for the satisfactory determination of type varies with the individual case, depending upon the abundance of pneumococci in the specimen of sputum and the virulence and invasive power of the strain present. It may be from 5 to 24 hours, averaging 6 to 8 hours with the parasitic fixed Types I, II, and III. As soon as the injected mouse appears sick a drop of peritoneal exudate is removed by means of peritoneal puncture with a sterile capillary pipette, spread on a slide, stained by Gram's method, and examined microscopically to determine whether there is an abundant growth of the pneumococcus present. If there is an abundant growth of the pneumococcus present in pure culture the mouse is killed and the determination of type proceeded with. If the growth is only moderate or if other organisms are present in any quantity, further time must be allowed until subsequent examination of the

peritoneal exudate shows an abundant growth of the pneumococcus. It should be emphasized that undue haste in killing the mouse is time lost in the end.

Mouse Autopsy.—As soon as the mouse is killed or dies, the peritoneal cavity is opened with sterile precautions and cultures are made of the exudate in plain broth and on one-half of a blood agar plate. Films are made and stained for microscopic examination by Gram's stain and Hiss's capsule stain. The peritoneal exudate is then washed out by means of a sterile glass pipette with 4 to 5 cc. of sterile salt solution, the washings being placed in a centrifuge tube. Cultures are then made from the heart's blood in plain broth and on the other half of the blood agar plate.

Determination of Type.

Agglutination Method.—When the pneumococcus is present in pure culture in the peritoneal exudate the determination of type may be satisfactorily made by macroscopic agglutination tests as follows. The peritoneal washings are centrifugalized at low speed for a few minutes until the cells and fibrin contained in the exudate are thrown down. The supernatant bacterial suspension is decanted into a second centrifuge tube and centrifugalized at high speed until the organisms are thrown down. The supernatant fluid is discarded and the bacterial sediment taken up in sufficient sterile salt solution to make a moderately heavy suspension. The concentration of bacteria should be similar to that of a good 18 hour broth culture of the pneumococcus. This suspension is used directly for macroscopic agglutination tests, being mixed with immune serum in small test-tubes in equal quantities of 0.5 cc. each.

To obviate the difficulty that occasionally arises from the occurrence of Group IV strains that show cross agglutination in all three types of immune serum, the optimum dilutions of serum and the optimum incubation time that will surely identify all type strains and fail to give any cross agglutination reactions have been determined on a large series of strains.[5] The results are shown in Table II.

[5] This work applies only to the antipneumococcus immune serum prepared at the Hospital of The Rockefeller Institute for Medical Research.

TABLE II.

Determination of Pneumococcus Types by Agglutination.

Pneumococcus suspension 0.5 cc.	Serum I (1:20) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:20) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I	++	−	−	−
" II	−	++	++	−
Subgroups II a, b, x	−	+	−	−
Type III	−	−	−	++
Group IV	−	−	−	−

Incubation for 1 hour at 37°C.

From Table II it will be seen that a 1:20 dilution of Type I serum, making with the addition of an equal amount of pneumococcus suspension a final dilution of 1:40, a 1:20 dilution of Type II serum making a final dilution of 1:40, and a 1:5 dilution of Type III serum making a final dilution of 1:10, serve to agglutinate Types I, II, and III pneumococci respectively and fail to show any cross agglutination reactions with strains belonging in Group IV. It will further be seen that with 0.5 cc. of undiluted Type II serum, as well as with the 1:20 dilution, pneumococci belonging to the various Subgroups II may be identified and rapidly differentiated from Type II pneumococci in that they show partial to complete agglutination in undiluted Type II serum, but not in the 1:20 dilution at the end of 1 hour's incubation at 37°C.

For the determination of types of pneumococci in the peritoneal washings such serum dilutions give the most satisfactory and clear-cut results. Five small test-tubes are set up as follows: Tube 1, 0.5 cc. of Serum I (1:20) + 0.5 cc. of bacterial suspension; Tube 2, 0.5 cc. of Serum II (undiluted) + 0.5 cc. of bacterial suspension; Tube 3, 0.5 cc. of Serum II (1:20) + 0.5 cc. of bacterial suspension; Tube 4, 0.5 cc. of Serum III (1:5) + 0.5 cc. of bacterial suspension; Tube 5, 0.1 cc. of sterile ox bile + 0.3 cc. of bacterial suspension to determine the bile-solubility of the strain for differentiation from the streptococcus. The tubes are incubated in the water bath for 1 hour at 37°C. Agglutination of Types I, II, and III pneumococci in such serum dilutions is practically always immediate in the homologous serum and no agglutination occurs in the heterologous sera.

Rapid clumping of the organisms is seen to take place and may be brought out clearly by gentle agitation of the tubes. For the identification of Subgroup II pneumococci incubation is necessary, such strains showing partial to complete agglutination in undiluted Type II serum at the end of 1 hour's incubation. If no agglutination occurs and the organism is bile-soluble, it is classified as a Group IV pneumococcus.

Precipitin Method.—It has been stated above that the determination of pneumococcus types by macroscopic agglutination tests with the peritoneal washings is interfered with when other organisms are present, with a resultant delay of 18 hours or more before the type of pneumococcus present can be established. To obviate this difficulty the following method has been devised. Dochez and Avery[6] have shown that the pneumococcus produces in broth cultures during the period of active growth a soluble substance which gives a specific precipitin reaction with the homologous antipneumococcus immune serum. It seemed probable that this soluble substance or precipitinogen would be present in the peritoneal exudate of the mouse in sufficient quantity to give a specific precipitin reaction with the homologous serum and such has proved to be the case. The method to be described is dependent upon this phenomenon.

The peritoneal exudate is washed out with 4 to 5 cc. of sterile salt solution by means of a sterile glass pipette and placed in a centrifuge tube. The peritoneal washings containing cells, fibrin, and bacteria are immediately centrifuged at high speed until the supernatant fluid is water clear. The supernatant fluid is then pipetted off, with care not to disturb the sediment, which is discarded, and is mixed in quantities of 0.5 cc. each with an equal amount of the antipneumococcus immune serum in a series of small test-tubes as follows: Tube 1, 0.5 cc. of Serum I (1:10) + 0.5 cc. of supernatant peritoneal washings; Tube 2, 0.5 cc. of Serum II (undiluted) + 0.5 cc. of supernatant peritoneal washings; Tube 3, 0.5 cc. of Serum II (1:10) + 0.5 cc. of supernatant peritoneal washings; Tube 4, 0.5 cc. of Serum III (1:5) + 0.5 cc. of supernatant peritoneal washings. An immediate specific precipitin reaction occurs in the tube contain-

[6] Dochez, A. R., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 75.

ing the homologous immune serum, the other tubes remaining clear (Table III). No incubation is necessary. Two tubes of Type II serum are used for the purpose of distinguishing between Type II pneumococci and members of the Subgroups II, the former giving a precipitin reaction in both tubes, the latter only in the undiluted Type II serum. A negative reaction in all tubes indicates a pneumococcus belonging in Group IV.

The method has been tested with a large number of strains and has been consistently positive and specific with pneumococci of Types I, II, and III and consistently negative with pneumococci of Group IV. The presence of other organisms together with the pneumococcus in the peritoneal exudate does not interfere with the reaction and other organisms than the pneumococcus produce no substance that might give a false positive reaction.

TABLE III.

Determination of Pneumococcus Types by the Precipitin Method.

Supernatant peritoneal washings 0.5 cc.	Serum I (1:10) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:10) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I	++	−	−	−
" II	−	++	++	−
Subgroups II a, b, x	−	+	−	−
Type III	−	−	−	++
Group IV	−	−	−	−

The results with Subgroup II pneumococci have not been so satisfactory. Reference to Table III will show that pneumococci belonging to these groups give a precipitin reaction with undiluted Type II serum but not with the 1:10 dilution, thereby being distinguished from Type II pneumococci. A number of Subgroup II organisms, however, have been encountered in which the peritoneal washings have failed to give a precipitin reaction with undiluted Type II serum. In the identification of the fixed parasitic types of pneumococci this occasional difficulty is of little practical importance from the point of view of treatment as there is at present no specific therapy for cases of pneumonia caused by pneumococci of the Subgroups II. For purposes of classification and statistics these organisms can be

readily identified subsequently when the organism has been obtained in pure culture.

In order to determine the shortest time after mouse inoculation in which a clear-cut positive precipitin reaction may be obtained and whether it occurs as soon as a satisfactory agglutination test can be made, a series of mice have been inoculated intraperitoneally with measured amounts of pure pneumococcus cultures and the mice have been killed at varying intervals. Simultaneous determinations of type have been made by both the agglutination and precipitin methods on each peritoneal washing. The results are shown in Table IV.

TABLE IV.

Comparison of Agglutination and Precipitin Methods.

Mouse.	Time killed after inoculation with 0.01 cc. of culture.	Series A, Type I.		Series B, Type II.		Series C, Type III.	
		Agglutination.	Precipitin.	Agglutination.	Precipitin.	Agglutination.	Precipitin.
	hrs.						
1	4	−	−	−	−	−	−
2	5	−	−	−	−	−	+
3	6	−	−	−	+	−	++
4	7	−	+	−	++	−	++
5	8	++	++	++	++	++	++

From these experiments it is evident that the agglutination method possesses no advantage in point of time over the precipitin method. The presence of the soluble precipitinogen in the peritoneal exudate in sufficient quantity to give a clear-cut precipitin reaction coincides in the case of Type I pneumococci with the earliest time when satisfactory agglutination tests can be made. With Type II pneumococci it occurs earlier and with Type III still earlier. This phenomenon exhibits an interesting parallelism with the capsular formation and virulence of the three types of pneumococci.

The precipitin method possesses the following distinct advantages. It is available as soon as satisfactory agglutination tests can be made; incubation of the tubes is unnecessary; it is not interfered with by the presence of other organisms in the exudate; it is specific and shows no cross immunity reactions; it is applicable to mice which

through unavoidable circumstances have been dead for some time before the determination of type can be made and in which autolysis of the pneumococci or postmortem invasion of the peritoneal cavity by other organisms has made the agglutination method impracticable. For these reasons it is recommended as the method of choice in all cases.

Identification of Type III Pneumococci by Morphological and Cultural Characteristics.—If Type III antipneumococcus immune serum is not available for diagnostic purposes Type III pneumococci may be identified in most instances by cultural and morphological characteristics. *Pneumococcus mucosus* is usually somewhat larger, rounder, and less lanceolate than other types of pneumococci. It possesses a large distinct capsule which stains readily with Hiss's capsule stain and usually retains the pink counterstain with Gram's method. The peritoneal exudate produced on mouse inoculation is usually quite mucoid and colonies on solid media are moist, mucoid, and spreading. It is always bile-soluble. These characteristics usually serve to differentiate Type III pneumococci from other types. Occasional strains of pneumococci which agglutinate in Type III serum, however, are encountered which do not show well developed mucoid characteristics and cannot be distinguished culturally from other types. Furthermore, Type II strains are occasionally met with that exhibit fairly well developed mucoid characteristics. For these reasons the identification of Type III pneumococci by morphological and cultural characteristics is not always absolute, and the diagnosis should be established by immunological methods when Type III serum is available.[7]

Confirmation of Type.

The determination of type on the peritoneal washings should be confirmed by macroscopic agglutination tests with a pure bouillon culture of the pneumococcus obtained from culture of the heart's blood at the time of mouse autopsy. The technique is the same as that employed in the agglutination tests on the bacterial suspension obtained from the peritoneal washings and should include a test for bile-solubility.

[7] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1917, xxv, 629.

Determination of Types of Pneumococci in Blood Cultures, Spinal Fluids, Empyema Fluids, and by Lung Puncture.

Blood Culture.—The usual technique in routine blood cultures is carried out. From a positive bouillon blood culture 10 cc. are removed by pipette and centrifugalized at low speed to remove the blood cells. The supernatant fluid is pipetted off and the bacteria are thrown down by centrifugalization at high speed, the supernatant fluid is discarded, and the bacterial sediment is suspended in sterile salt solution. The pneumococcus type is then determined by macroscopic agglutination tests following the same technique described above.

Spinal Fluid and Empyema Fluid.—Cultures are made by the methods ordinarily employed in culturing fluids and the type of pneumococcus is determined when the culture has grown out by the use of the same technique as that applied to blood cultures. If desired, in addition to culturing spinal fluids, a portion of the fluid may be centrifugalized at high speed to throw down the pneumococci present, and the sediment, taken up in 1 cc. of sterile salt solution, inoculated intraperitoneally into a mouse.

Lung Puncture.—This procedure should be resorted to only when it is impossible to obtain a suitable specimen of sputum or a positive blood culture. Cultures are made in bouillon of the lung puncture material and the determination of type is made by the same technique as that employed in the case of blood cultures.

Determination of Pneumococcus Types by Direct Sputum Culture.

It was thought that the determination of types of pneumococci by the precipitin method might be possible by direct culture of the washed sputum in bouillon without resort to mouse passage. Tubes of bouillon were inoculated with specimens of sputum and incubated 6 to 8 hours at 37° C. The cultures were then centrifugalized at high speed until the supernatant bouillon was clear, and precipitin tests were made by mixing equal parts of the supernatant bouillon and immune serum. In a few instances sufficient precipitinogen had been produced by the growth of the pneumococcus in the culture to give a positive precipitin reaction with the homologous serum. This by

no means invariably occurred with all specimens of sputum, however, and seemed to depend largely upon the number of pneumococci present in the sputum and to some extent upon the type of pneumococcus. The number of experiments done was small and the results were not sufficiently satisfactory to make the method of practical use. It is possible that further experiments along these lines might develop a technique which would prove available for use as a routine method.

SUMMARY.

The determination of pneumococcus types in lobar pneumonia is of value in the field of prognosis and as a prerequisite for specific serum therapy. The method for the determination of types should be as rapid as possible and a standard technique should be employed. The most satisfactory method is by the intraperitoneal inoculation of a mouse with the patient's sputum, by which means a rapid and abundant growth of the pneumococcus is obtained and secondary organisms are rapidly eliminated in most instances. The diagnosis of type is made directly on the peritoneal exudate. Certain factors in the method commonly used have interfered with the rapid determination of types in an appreciable number of cases, notably the growth of other organisms in the peritoneal exudate together with the pneumococcus, and some confusion has arisen because occasional strains of pneumococci have been encountered that show cross agglutination reactions when undiluted immune serum is used. Such reactions have been shown to be due to a limited zone of non-specific immunity and they in no way invalidate the classification of the pneumococci into sharply defined immunological groups. The optimum dilutions of serum have been determined that will agglutinate all type strains of pneumococci and fail to cause any cross agglutination reactions when mixed with equal amounts of pneumococcus cultures and incubated for 1 hour at 37°C. They are a 1:20 dilution of Serum I, a 1:20 dilution of Serum II, and a 1:5 dilution of Serum III. For the diagnosis of Subgroup II pneumococci undiluted Type II serum is required.

To obviate the other difficulties of the method commonly used a new method for the determination of types has been devised. It

depends upon the fact that there is produced by the growth of the pneumococcus a soluble substance which is present in the peritoneal exudate of the mouse in sufficient quantity to give a specific precipitin reaction with the homologous immune serum. The precipitin method can be used in all instances in which the determination of types by the agglutination method is possible, and it possesses certain distinct advantages which make it available when the agglutination method is impracticable. It is of particular value as a time-saving device in those instances where the presence of other organisms together with the pneumococcus in the peritoneal exudate causes a delay of 18 hours or more before the type of pneumococcus can be definitely established. It is therefore recommended as the method of choice in all cases. If desired, both the agglutination and precipitin methods may be applied to the same specimen of peritoneal washings.