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Influence of Type and Concentration of Antitoxin on the *in vitro* Toxigenicity Test for *C. diphtheriae*

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The *in vitro* method for determining the toxigenicity of *Corynebacterium diphtheriae* cultures was introduced in 1948 simultaneously by Elek (*1*) in Britain and Ouchterlony (*2*) in Sweden. Both authors have elaborated on their original descriptions (*3, 4, 5*). King et al. (*6*) in this country reported favorably on Elek's test and introduced several modifications.

Many variables are concerned in the *in vitro* reaction. Elek's recent paper (*3*) described the effect of various changes in the composition of the medium. He showed that the brand of lactic acid and the type of peptone used may materially affect the toxin-antitoxin reaction. In the same report, Elek demonstrated that the presence of natural diphtheria antitoxin in serum added for medium enrichment can interfere with production of the specific *in vitro* arrowhead precipitate. This result would seem to justify the exercise of caution in the use of human serum recommended by Carter and Wilson (*7*). Although the latter authors do not record any discrepancies in their results, King et al. (*6*) found human serum inferior to horse, rabbit, and sheep sera.

Before the *in vitro* toxigenicity test can be adopted as a routine public health laboratory procedure, another variable must be considered, namely, the specific diphtheria antitoxin used to soak the filter paper strip. The commercial antitoxins which are available were designed for therapeutic use, and the biologic firms manufacturing them make no claim to an antigenically monovalent product. As a consequence, the content of antibody other than antitoxin may be expected to vary in the different products. Such variation is indicated by the data recorded below.

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Materials and Methods

Field cultures that had been classified as *C. diphtheriae* according to the criteria of Anderson et al. (*8*) were used. In the experiments in tables 2 and 3, culture No. 1 was a gravis strain, No. 7 was a diphtheroid, and Nos. 2 to 6 and Nos. 8 to 11 were atypical mitis strains. The *in vitro* method followed was that of Elek (*1*) as modified by King et al. (*6*). The pH of the medium after sterilization and prior to addition of the serum was adjusted to 7.8. Sheep serum was employed as the enriching agent. The commercial antitoxin was diluted to 500 units per ml. (on the basis of the labelled unitage) in 0.85 percent NaCl solution. The test plates were inoculated from 24-hour brain-heart infusion broth cultures, and guinea pig intracutaneous toxigenicity tests were conducted with the same broth culture material. The plates were incubated at 37° C. for 3 days. Readings were made at 24-hour intervals during incubation, with a final reading at 72 hours. The plates were then kept at room temperature or in the refrigerator for an additional 7 days following the final reading. Although the reactions increased progressively after the third day, there were no significant qualitative changes.

Experimental

Production of Arrowhead Precipitates by Avirulent Strains

Previous investigations (*3*, *6*, *7*) have established complete correlation between positive *in vivo* and *in vitro* toxigenicity tests when the tests are read within 48 to 72 hours. In the course of checking the toxicity of our *C. diphtheriae* culture collection by the *in vitro* method, a number of arrowhead precipitates were recorded for the plate test within 72 hours on strains that previously were found to be avirulent [1] in animals (table 1).

These *in vitro* positive, *in vivo* negative strains, which were in all respects *C. diphtheriae*-like except for animal toxigenicity, were isolated from clinically diagnosed diphtheria patients from whom no toxigenic cultures could be obtained. Repeated virulence tests with such strains in guinea pigs, by intracutaneous, subcutaneous, and intraperitoneal routes, and the intracutaneous route in rabbits were interpreted as negative, even though occasional unprotected skin reactions were slightly greater than control reactions. Confirmation of the avirulence of these cultures was obtained from two independent laboratories.[2] Such cultures would seem to be very similar to those described by Frobisher et al. (*9*) in their study of the virulence of nontoxigenic *C. diphtheriae* strains.

[1] The terms virulent and avirulent are used only in reference to the property of toxigenicity in animals.

[2] Public Health Laboratory, Seattle, and Division of Laboratories, California State Department of Public Health.

Table 1. *Comparison of in vitro and in vivo toxigenicity tests on field cultures from diphtheria cases and contacts*

Cultural classification	*In vitro* [1]	*In vivo* [2]	Number of strains
C. diphtheriae	+	+	143
	0	0	10
	+	0	[3] 11
	0	+	[4] 10
Diphtheroids	0	0	33
Non-Corynebacterium	0	0	10

[1] *In vitro* toxigenicity plate test as described in text.
[2] Intracutaneous guinea pig virulence test.
[3] 4 of these cultures were typed atypical gravis and the remaining 7, atypical mitis. These strains are represented in tables 2 and 3 by culture numbers 2, 3, 5, 6, 8 and 10.
[4] All of these strains were intermedius in type. It was therefore assumed that the negative *in vitro* results were due to traces of hemoglobin in the sheep serum—see King et al. (*6*).

The positive *in vitro* reactions recorded for these avirulent strains were reproduced repeatedly, always within the first 72 hours of incubation. The precipitates produced by the avirulent cultures were usually not as well defined as the primary arrowhead precipitates of typical virulent cultures (figs. 1 and 2). Such reactions unquestionably are the same as the "false toxicity reactions" of Ouchterlony (*4*,*5*) and the "secondary lines" of Elek (*3*). In reference to the secondary precipitates, Elek warned: "To avoid confusion the plate test must not be read later than 48 hours." In our experience, such a time restriction has been undesirable. *In vivo* positive cultures not infrequently showed delayed production of the toxin-antitoxin precipitate. Since it would be preferable to allow slow toxin-producing strains ample incubation time, methods of completely eliminating the secondary reactions were investigated.

Comparison of Different Antitoxin Products

If the secondary precipitates are the result of other antigen-antibody interactions, one would not necessarily expect to get identical results when using different brands of commercial antitoxin. To determine whether or not variation did occur, five different brands of commercial antitoxin were purchased and diluted to 500 units per ml.[3] The same lots of medium and sheep serum were used throughout, and the same broth cultures were employed to inoculate all five plates in the series. The results are recorded in table 2.

[3] In the text following, A = Eli Lilly & Co. brand of antitoxin; B = E. R. Squibb & Sons; C = Cutter Laboratories; D = Parke Davis & Co.; and E = Lederle Laboratories brand. The author wishes to emphasize that, in the present state of our knowledge concerning secondary *in vitro* precipitates, the comparison of different antitoxin brands is in no way indicative of the superiority of any one product over the others.

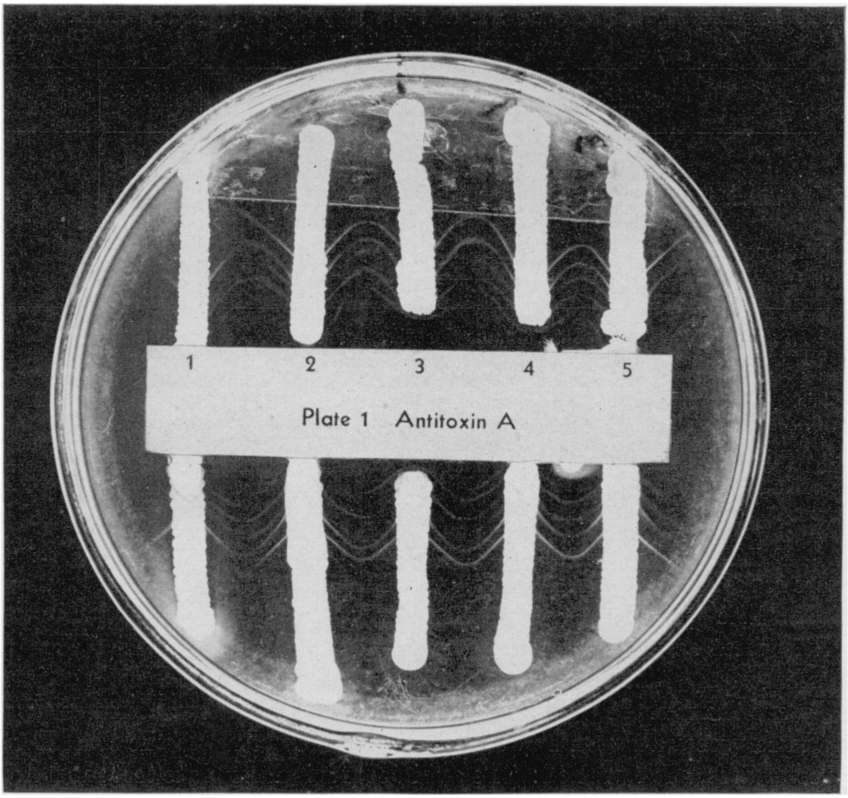

Figure 1. Culture 1 was avirulent and 2–5 were virulent when tested on animals. Note the presence and fusion of primary toxin-antitoxin lines in cultures 2–5 and their absence in culture 1. All the cultures have produced secondary lines.

Table 2. *Effect of different commercial antitoxins on the occurrence of primary and secondary precipitates in the in vitro toxigenicity test*

Culture No.	Animal test	Brand of commercial antitoxin (500 units /ml.)				
		A	B	C	D	E
1	Positive	[1] 4+s	4+	4+	4+	4+
2	Negative	2+	0	1+	0	0
3	do	2+	0	2+	±	0
4	do	0	0	0	0	0
5	do	2+	0	2+	0	0
6	do	1+	0	1+	0	0
7	do	0	0	0	0	0
8	do	1+	0	0	0	0
9	Positive	4+s	4+	4+s	2+s	3+
10	Negative	1+	0	1+	0	0

[1] 0 to 4+ indicates the size and clarity of the linear arrowhead precipitates; "s" indicates appearance of secondary lines in addition to a primary precipitate. Plates read after 72 hours at 37° C.

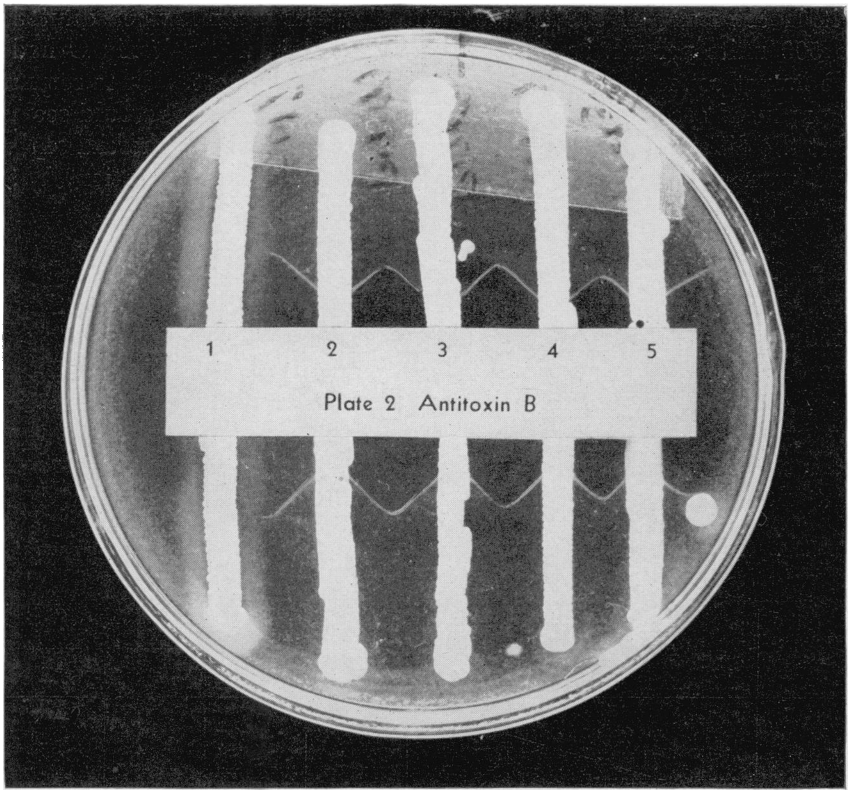

Figure 2. Plates 1 and 2 inoculated at the same time with the same cultures. Only the brand of antitoxin has been changed. Only primary lines are now visible, and therefore culture 1 shows complete absence of linear precipitates. Both plates incubated 72 hours at 37° C. (Photographs courtesy G. W. Reis, Department of Medical Photography, University of Washington.)

Commercial serum A was the brand which had been used originally. Serum C is remarkably similar to A in its reactions. Brands B and E did not show any secondary lines. Antitoxin D showed a slight arrowhead precipitate with an avirulent culture. The typical virulent cultures showed characteristic primary linear precipitates with all five brands of antitoxin.

Consideration was given to the possibility that these differences in commercial antitoxins might be due to a variation in concentration of the serum rather than to a qualitative difference in composition. Such a variation could result from inclusion of more antitoxin than the amount indicated on the label in order to allow for deterioration. As there is no theoretical limit to this excess, the products might be expected to vary in true antitoxin concentration. Rough flocculation tests were conducted according to the method of Glenny and Okell (*10*). All five products were found to have an antitoxin concentration within the range of 500 to 800 antitoxic units per ml. when diluted to 500 units per ml. as determined by the label. Antitoxin C had the lowest concentration and antitoxin E, the highest. Since serum C produced secondary reactions and serum E did not, it would seem that the differences in reaction produced by the five commercial antisera were not the result of variation in serum concentration, but rather of variation in serum constitution.

Effect of Diluting Antitoxin

Ouchterlony (*4*, *5*) had noted that the false toxicity reactions produced by his technique could be reduced appreciably by decreasing the concentration of antitoxin. To determine the effect of dilution on the five different products, all samples were diluted to a concentration of 50 units per ml. and the tests repeated with seven of the cultures used in the previous experiment. The results are recorded in table 3.

Table 3. *Effect of dilution of antitoxin on occurrence of primary and secondary precipitates in the in vitro toxigenicity test*

Culture No.	Animal test	Brand of commercial antitoxin (50 units/ml.)				
		A	B	C	D	E
1	Positive	[1] 4+	3+	2+	3+	3+
2	Negative	0	0	0	0	0
4	do	0	0	0	0	0
5	do	0	0	0	0	0
6	do	0	0	0	0	0
8	do	0	0	0	0	0
10	do	0	0	0	0	0
11	Positive	3+	2+	2+	2+	2+

[1] 0 to 4+ indicates the size and clarity of the linear arrowhead precipitates There were no secondary lines associated with the positive reactions recorded above. Plates read after 72 hours at 37° C.

Dilution of all commercial antitoxins tested caused the secondary lines to disappear, but at the same time allowed the primary precipitates to remain evident. In another experiment, the concentration of antitoxin A was decreased from 2,000 units per ml. to 62.5 units per ml. by serial doubling dilution. The secondary reactions of both virulent and avirulent cultures progressively disappeared in direct proportion to the decreasing antitoxin concentration.

Discussion

Before the *in vitro* toxigenicity test for *C. diphtheriae* can be accepted as a routine public health laboratory procedure, all known variables must be adequately controlled. In addition, the significance of all possible reactions should be understood. In the animal toxigenicity test (unfortunately known as the "virulence" test in common laboratory parlance) a reaction is read as positive if the characteristic lesion which appears in the absence of specific antitoxin is prevented in its presence. The occurrence in commercial antitoxin of other components reacting specifically with the diphtheria bacillus or its products presumably does not interfere with the interpretation of such toxicity reactions. However, in the *in vitro* test, where the visible interaction of antigen and antibody is the basis for a positive result, discrepancies can readily arise.

As the described experiments have demonstrated, the secondary reactions can be avoided either by the selection of a commercial antitoxin that fails to produce such precipitates or by suitable dilution of an antitoxin that does produce them. If the latter method is adopted, the diluted serum must be checked carefully with control positive and negative cultures.

Although only single lots of five commercial antisera were tested, the evidence obtained would seem to justify further investigation with the objective of providing a standard purified antitoxin for laboratory use only. If the secondary reactions are proved to be antigenic, a purified antitoxin could be prepared either by absorption of the crude product with avirulent culture material or by use of a purified toxin as the stimulating antigen in the process of antitoxin production. Lawrence and Pappenheimer (*12*) already have demonstrated the feasibility of producing a diphtheria toxin free of an atoxic antigenic component which they called the P-fraction. Whether or not this P-fraction and the secondary precipitates of the *in vitro* test are related remains to be established.

Although secondary reactions may be regarded as a nuisance to laboratory personnel required to report on the virulence of field cultures, the strains producing them present an extremely interesting relationship to typical toxigenic *C. diphtheriae* cultures. Diphtheria investigators have long argued the status of avirulent diphtheria organisms. Because there is no good evidence that these strains are able to become toxigenic for animals, their public health significance remains obscure. Frobisher et al. (*9, 11*) have attempted to demonstrate diphtheria immunizing powers of avirulent cultures when tested in mice and rabbits. Although not conclusive, their results certainly warrant further investigations. Observations with the *in vitro* technique may contribute materially to such research.

Summary

1. The production of arrowhead precipitates by avirulent *C. diphtheriae* cultures in less than 72 hours on *in vitro* toxigenicity test plates were described.

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2. Five different brands of commercial antitoxin were compared in the *in vitro* test to determine the variability of production of secondary precipitates. All five produced primary lines with virulent cultures, but two failed entirely to show the secondary reactions.

3. Dilution of the antitoxin used in the plate test resulted in the disappearance of secondary precipitates produced by both virulent and avirulent cultures. The characteristic primary precipitates persisted at a dilution of 50 units per ml.

4. The production of secondary lines by the commercial antitoxin products showed no correlation with the true antitoxin concentration of the different brands, a fact which was interpreted as signifying a qualitative difference in the components responsible for the secondary reactions.

ACKNOWLEDGMENT

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A Study of Certain Factors Affecting the Agglutination Test for Brucellosis

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The agglutination test for brucellosis is much used as a diagnostic procedure and should be as fully standardized as possible. Comparative studies by other workers have revealed considerable divergence in methods and results of tests in different laboratories (*1*). In view of this fact and of the data obtained from a questionnaire distributed by the National Research Council Committee on Brucellosis (*2*), it has become obvious that further efforts to evaluate these procedures and standardize the test are desirable.

The present study was undertaken to compare procedures now in use for the agglutination test for brucellosis and to evaluate various modifications of technical factors in the test so that a standard and reliable procedure could be established.

Experimental

A collection of positive *Brucella* sera was obtained from Dr. B. N. Carle of the National Institutes of Health, Dr. D. L. Lichty of Wisconsin, and Dr. M. Ruiz Castaneda of Mexico, and several State health laboratories. It was decided to use the maximum titer obtainable as the criterion of sensitivity for each of the test methods examined. The maximum titer of the serum (as given in this report) represents the highest dilution of serum which gave complete agglutination of the antigen, accompanied by a perfectly clear supernatant fluid.

The factors involved in the agglutination reaction which were investigated in different parts of the study were:

1. Antigen preparation and incubation conditions.
2. Antigen strain and media used for antigen production.
3. Effect of pH on the agglutination titer.

Materials and Methods

Part I: Antigen Preparation and Incubation Conditions

This part of the investigation involved a study of 105 sera—70 from cows, 5 from goats, and 7 from persons. A strain of *Brucella*

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was isolated from each donor of serum, but data on the strain isolated were not available.

The antigen used as a preliminary basis for comparison was made by the Bureau of Animal Industry, Beltsville, Md., from *Brucella abortus*, strain 1119–3, and was tested in accordance with methods developed by the Bureau of Animal Industry (*3*) for the *Brucella* agglutination test in test tubes.

In preparing our own antigen, the organisms of this strain were harvested in phenolized physiological saline and examined for purity. The suspension was filtered through a thick pad of sterile, nonabsorbent cotton into a suction flask with the aid of a vacuum. The filtered suspension was heated in flowing steam for 25 minutes, then it was centrifuged and the supernatant fluid discarded. This procedure departs from the Bureau of Animal Industry method in that the antigen suspension is centrifuged before heating. The cells were resuspended by vigorous agitation and diluted to obtain nine different antigen densities. The density of the antigen for each test was separately adjusted by setting up the McFarland nephelometer standards (also known as barium sulfate standards). The standards were always prepared immediately before using. The degrees of turbidity were read by means of the Lumetron photoelectric colorimeter.

Tubes used in the agglutination studies measured 13 by 100 mm. Twofold dilutions of serum of from 1 in 25 to 1 in 12,800 were prepared in the tubes with the diluent.

In a series of preliminary tests, nine different methods of antigen preparation and four different incubation conditions were tested with 20 sera.

The tests were read at 24 and 48 hours, parallel series being incubated in the water bath at 37° C. and at 52° C., in the dry incubator at 37° C., and in the water bath at 52° C. for 4 hours followed by storage in the icebox at 4° to 6° C. to complete the incubation. The water baths were of the gable-covered type.

The nine antigens and procedures were as follows:

TEST A. This was carried out with antigen obtained from the Bureau of Animal Industry and tested according to their method (*3*). The antigen concentration was equivalent to a barium sulfate standard tube No. 1. Dilutions of serum were made by adding 4 ml. of antigen to the first tube and 2 ml. to nine other tubes. Sixteen-hundredths milliliter of serum was added to the first tube, yielding a dilution of 1: 25, and this was further diluted by mixing 2 ml. in series with the antigen in the other tubes.

TEST B. The antigen used in test B and all following tests was prepared in this laboratory as described above. The procedure for test B was the same as for test A. The antigen density was equal to a barium sulfate standard No. 1.

TEST C. The antigen density for test C was equal to a barium sulfate standard No. 2. In this procedure, 2 ml. of saline was placed in the first tube and 1 ml. of saline in the remaining nine tubes. Sixteen-hundredths milliliter of serum was added to tube one and then serially diluted in the usual manner. One milliter of antigen was then added to each of the 10 tubes of diluted serum.

TEST D. Antigen for test D was adjusted to a barium sulfate standard of 1.5 and tested as described in test A in which the antigen was used as the diluent.

TEST E. Antigen for this test was adjusted to a barium sulphate standard No. 3 and tested as described in test C, using saline as the diluent.

TEST F. The antigen for test F was adjusted to a barium sulfate standard No. 3 and tested as described in test A in which the antigen was used as the diluent.

TEST G. In test G the antigen was adjusted to a barium sulfate standard No. 6. Serum was tested as in test C in which saline was used as the diluent.

TEST H. In this test, the antigen was adjusted to a barium sulfate standard No. 1 and tested as described in test C in which saline was used as the diluent.

TEST I. In this, the last test, the antigen was adjusted to a barium sulfate standard No. 1.5, and the procedure was as in test C in which the saline is the diluent of the serum.

The initial density of antigen in tests C, E, G, H, and I, was halved when the antigen was added to the serum-saline mixtures.

Part II: Antigen Strain and Media Used for Antigen Production

Three cultures of *Br. abortus:* strain 1119–3 from the Bureau of Animal Industry, strain 1336 sent by Dr. I. F. Huddleson, and strain 456 from the National Institutes of Health were compared. For comparative purposes, each was cultivated for antigen on potato-infusion 3 percent agar (the Bureau of Animal Industry Medium) and on tryptose 3 percent agar. The formulas follow:

Potato-Infusion 3 percent Agar

Potato infusion	1000 ml.
Agar	30 gm.
Bacto-peptone or equivalent	10 gm.
Beef extract, Liebig's or equivalent	5 gm.
Sodium chloride U. S. P	5 gm.
Glycerine U. S. P	20 ml.
Dextrose U. S. P	10 gm.
Final pH	6.4–6.8

Tryptose 3 percent Agar

Bacto-tryptose	20 gm.
Bacto-dextrose	1 gm.
Sodium chloride	5 gm.
Bacto-agar	30 gm.
Final pH	6.9

The resulting six antigens were tested with 83 sera from known cases of brucellosis. The strains and the media used were the only variables compared. The method of test C as described above, with incubation in the water bath at 52° C., was used in this comparison.

Part III: Effect of pH on the Agglutination Titer

In this part of the study, Clark buffers (*4*), pH 5, 6, 7, 8, and 9, were prepared as diluent for the serum. Sera from 44 known cases of brucellosis were tested. The technique of test C was used in parallel series of tests. In one series of tests, the sera were diluted with isotonic saline solution. In the other series, the sera were diluted with the buffered saline solutions which were adjusted so that the total salt concentrations of the buffered solutions were as nearly isotonic as possible. A determination of the pH of 1 ml. of each buffer solution when diluted with 1 ml. of antigen showed that pH 5.0, 6.0, 7.0, 8.0, and 9.0 changed to pH 4.9, 5.8, 6.8, 7.7, and 8.6, respectively. The pH of saline, which was 6.0, became 6.2 when diluted with antigen. The pH of the antigen was 6.7. The agglutination test was studied with these final pH values.

Method of Reading All Tests and Noting Results

The tubes were held up to a fluorescent light and read against a dark background. A positive agglutination was denoted by agglutinated cells in a sparkling, clear, supernatant liquid. An incomplete agglutination was noted when agglutinated cells were viewed in a cloudy supernatant liquid. A negative result was obtained when no agglutination was visible.

Results—Part I

The *Brucella* agglutination titers obtained with tests A–G, using 20 sera in preliminary studies, were tabulated. Tests H and I were compared with test C separately and are discussed later. The number of times any one test gave the highest titer when compared with the other six tests was noted. The summarized data on the first 20 specimens showed clearly that, irrespective of the times or temperature of incubation, test C gave better results than any of the other six tests. Tests B and E were better in the order named than tests A, D, G, or F. The results also showed that incubation at 52° C. for 4 hours, followed by refrigeration, generally yielded lower titers than continual incubation at 52° C. There was little choice between 37° C. dry, and 37° C. water bath incubation. However, the 37° C. dry incubation is a variable incubation condition and the fluid in the tubes reach incubator temperature slowly, depending on the incubator load and other factors. Both the 37° C. dry incubation, and incubation at 52° C. for 4 hours, followed by refrigeration, as well as tests A, D, F, and G, were discontinued.

The comparison of tests B, C, and E, using incubation at 37° C. and 52° C. in the water bath, was continued until 105 specimens were tested. The results again were analyzed and numbers of sera yielding higher, equal, or lower titers with each antigen were computed. Due to the complexity of the data, only the summarized results are given. The results revealed that, with test C in 24 hours, 33 sera yielded higher titers at 52° C. than at 27° C., while 50 sera gave equal titers at both temperatures, and 22 sera yielded lower titers at 52° C. At the 48-hour interval, 31 sera yielded higher titers at 52° C., while 53 gave equal titers at both temperatures, and 21 sera gave lower titers at 52° C. Thus, there were no significant differences between the results read after 48 hours and those obtained in 24 hours, and no marked influence of temperature, although 52° C. seemed to favor higher titers. In addition, examination of the tests earlier than 24 hours showed that the agglutination process was proceeding faster in the 52° C. water bath. Tests B and E yielded concordant results with generally lower titers. Accordingly, all further tests were carried out in the water bath at 52° C., and readings were recorded after 24 and 48 hours. However, only readings at 24 hours are discussed in this report.

An analysis of results obtained with tests B, C, and E incubated at 52° C. for 24 hours showed that test C gave 62 higher titers, 41 equal titers, and 2 lower titers than B; that test C gave 55 higher titers, 44 equal titers, and 6 lower titers than E. Test E gave 29 higher titers, 65 equal titers, and 11 lower titers than B. These figures clearly indicate the superiority of test C over E or B. Test E was better than test B.

Tests H and I were set up for the purpose of evaluating antigen densities lower than those commonly in use. These were compared with test C on a total of 37 specimens using water-bath incubation at 52° C. for 24 hours.

An analysis of these results showed that test H gave 31 higher, 5 equal, and 1 lower titers than test C; and 20 higher titers, 12 equal, and 5 lower titers when compared with test I. Test I gave 10 higher titers, 25 equal, and 2 lower titers than test C. The results indicate that, with these 37 specimens, test H was superior to I and C. Test I was better than test C. This suggests that the more dilute the antigen, within range of good visibility, the more sensitive the test.

Results—Part II

The effectiveness of the three strains of *Br. abortus* described under "Materials and Methods" was compared when cultivated on potato-infusion agar, and on tryptose agar. A total of 83 positive sera, of which 24 were bovine, 33 human, 12 simian, 13 caprine, and 1 porcine, were tested in this comparison.

Readings were made after 24 hours incubation in the water bath at 52° C. The antigens were prepared and tested according to test C.

The results in the table show that *Brucella* strain 1336 cultivated on potato agar was a better antigen than strain 1119–3 cultivated on either potato or tryptose agar. Strain 1119–3 was inferior to strains 1336 or 456 irrespective of the media used. Higher titers were

Comparison of three antigen strains cultivated on potato and tryptose agar, tested with 83 sera; 24-hour reading

Of 83 sera tested with antigen	Those noted below gave titers			When compared with antigen
	Higher	Equal	Lower	
1119–3 P*	26	50	7	1119–3 T
1119–3 P	3	57	23	1336 P
1119–3 P	8	59	16	456 P
1336 P	22	49	12	456 P
1336 P	27	45	11	1336 T
1119–3 T**	7	56	20	1336 T
1119–3 T	3	33	47	456 T
1336 T	3	43	37	456 T
456 T	25	53	11	456 P
456 T	30	35	18	1336 P

* Potato-infusion agar.
** Tryptose agar.

obtained with strain 1119–3 cultivated on potato-infusion agar than when cultivated on tryptose agar. Strain 456 cultivated on tryptose agar gave more higher titers than strains 1336 and 1119–3 cultivated on tryptose or potato-infusion agar.

Results—Part III

The results obtained with diluents buffered at pH 5 and 6 were inferior to those obtained at pH 7. pH 5, compared with pH 7, gave 2 higher, 14 equal, and 28 lower titers. A comparison of pH 6 with pH 7 showed 8 higher, 24 equal, and 12 lower titers with pH 6. The results obtained with pH 8 and 9 were also inferior to pH 7. pH 9, compared with pH 7, gave 2 higher, 31 equal, 11 lower titers. A comparison of pH 8 with pH 7 showed 5 higher, 31 equal, and 8 lower titers with pH 8. The results with saline (pH after addition of antigen was 6.2) were equal to those with pH 6.0 buffer; in this case, 10 higher, 24 equal, and 10 lower titers were obtained with saline. These data indicate that the pH of the diluent should be near neutrality.

Discussion

The results show that antigen for test B, made in this laboratory, was more sensitive than the antigen for test A prepared by the Bureau of Animal Industry in Beltsville, Md. It is possible that heating the antigen before centrifugation increased the sensitivity of the preparation.

Comparison of tests in which the antigen was the diluent—tests B, D, and F—show that lower titers were obtained than when saline was the diluent—tests C, E, and G—irrespective of the antigen density. This suggests that serum should be diluted with saline and not with the antigen.

Test H, with an antigen density equivalent to McFarland No. 1, was superior to heavier antigen densities. This was especially true with low-titer sera. Tests made with antigens having densities lower than McFarland No. 1 were not satisfactory because the antigen was too dilute to read.

The McFarland nephelometer or barium sulfate standards may be a source of considerable error. Readings made with the Lumetron photoelectric colorimeter showed decreasing translucence with aging of the standards. This was not detected easily by the unaided eye. However, changes in translucence were not observed to occur in bacterial suspensions.

Most of the serum specimens exhibited their maximum titer in 24 hours. Although 48-hour results are not recorded, an occasional specimen required incubation for 48 hours before the agglutination was complete.

The results found indicate that continual incubation at 52° C. was superior to incubation for 4 hours followed by storage in the refrigerator for the remaining 20 hours. The results obtained by Henry and Traum (*5*) also showed that continual incubation was superior to incubation and storage in the icebox. However, they made their tests at 37° C. instead of 52° C.

According to the results obtained in this study, *Brucella* strain 456, obtained from the National Institutes of Health, was the best antigen when cultivated on tryptose agar. Strains 1119–3 and 1336 were better antigens when cultivated on potato-infusion agar than when cultivated on tryptose agar.

The agglutination did not proceed well on the acid or alkaline side of neutrality. Saline made with neutral distilled water or buffered at pH 7.0 would be preferable for the agglutination test.

Summary and Conclusion

Certain factors affecting the *Brucella* agglutination test in tubes were studied on 269 sera from known cases of brucellosis. Under the experimental conditions described in this report:

1. Antigen density equivalent to barium sulfate standard No. 1 gave the highest titers.

2. The dilution of serum with saline is preferable to dilution with antigen.

3. *Br. abortus,* strain 456, obtained from the National Institutes of Health and cultivated on tryptose agar gave more higher titers than did strain 1119–3 obtained from the Bureau of Animal Industry, or 1336 obtained from Dr. I. F. Huddleson and cultivated on either tryptose or potato agar.

4. Better results were obtained when the agglutination tests were incubated continuously in the water bath at 52° C. for 24 hours than when incubated at 37° C. or when refrigerated.

5. Higher agglutinin titers are exhibited by a few sera if readings are made at 48 hours rather than 24 hours.

6. The agglutination reaction operates best near neutrality.

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(*5*) Henry, B. S. and Traum, J.: A comparison of factors influencing the agglutination test for *Brucella abortus*. J. Infect. Diseases **47:** 367–379 (1930).

Stability of the Rh Factor in Mailed Samples

By Clifford I. Argall*

When the Utah State Department of Health's prenatal Rh typing program began in 1947, one of the major unanswered problems concerned the reliability of mailed samples for blood grouping. No satisfactory evidence was available, but other laboratories had indicated that such specimens were probably satisfactory. It was decided that the program should be started without delay, but that a controlled investigation would be made at a later date to determine exactly what percentage of error could be expected as a result of any adverse conditions in the ordinary mail. The data obtained may be of value to other laboratories engaged in or planning similar programs.

The 1,417 specimens received in this laboratory for routine prenatal testing were used in this study, 1,099 being Rh positive and 318 Rh negative. (The term, Rh positive, is used for erythrocytes containing the D antigen, and Rh negative, for those lacking it.) All were in excellent condition upon arrival. They were centrifuged at 4,200 rpm for 5 minutes and the serum poured off for use in the serological test for syphilis. Two percent suspensions of the cells were made in 0.85 percent saline, washed, resuspended, and the Rh type determined by the tube method. The slide test was also done, using a heavy

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cell suspension direct from the clot. After the results had been recorded, the vials containing the clots were packed and mailed to our branch laboratory at Cedar City, Utah, a distance of 263 miles. The time in transit varied from 1½ to 3 days. Without being unwrapped, they were returned to us by mail. The Rh typing was repeated, but only the tube test was used. So many samples showed such an advanced state of hemolysis that it was impossible to get a cell concentration heavy enough to obtain reliable results with the slide test.

Only one of the 1,417 samples studied showed a discrepancy in typing. This lone specimen, typed weakly positive originally, was Rh negative when repeated. Exactly what factor or factors influenced this result cannot, of course, be determined. The only consideration in this study was the over-all effect upon the Rh factor of varying conditions in the mail, so no specific cause can be assigned. It would seem very likely that an intrinsic weakness in the antigenic structure of those particular erythrocytes or an actual laboratory error in the typing would be the answer, since none of the other samples were affected in any way by the same conditions. The incidence of error, 0.07 percent, is almost the same as for our routine work, 0.09 percent, and does not represent any significant difference.

In order to show that climatic conditions produce no specific effect, 797 of the samples tested were sent during the coldest months of the year, January and February, and the remaining 620, during the last of June and part of July. The only discordant result occurred in the former group. Neither winter cold nor summer heat seemed to produce any effect on the antigen, but as might be expected, the amount of hemolysis was sharply increased during the hot months (table 1).

Since hemolysis has been frequently mentioned as a possible cause for unreliable typings, the specimens were divided very roughly into four groups (see table 1):

Very badly hemolyzed. Clots were shiny and irridescent. Very thin cell suspensions could be made, but only with difficulty. Practically all showed the purplish discoloration of bacterial contamination.

Badly hemolyzed. All suspensions could be made fairly easily, but when washed, the supernatant was deep red in color.

Hemolyzed. Supernatant from washing was a light, but definite red.

Slightly hemolyzed. Supernatant from washing showed only a faint pink.

No correlation whatever between hemolysis and inaccuracy in typing could be observed. In fact, the single erroneous result came in the hemolyzed group.

No attempt was made to identify the contaminating organisms in those specimens showing obvious growth. In this study, bacteria had no effect on the results, although it is possible that some bacteria might be able to destroy the antigen.

Table 1. *Classification of mailed samples on the basis of hemolysis*

Degree of hemolysis	Winter		Summer		Total
	Number of samples	Percent	Number of samples	Percent	
Very badly hemolyzed	223	28.0	267	43.0	490
Badly hemolyzed	166	20.7	201	32.4	367
Hemolyzed	264	33.2	95	15.2	359
Slightly hemolyzed	144	18.1	57	9.4	201

We were rather surprised to find that the age of the cells made no difference, up to the limits of this study. Table 2 gives the age of the cells used, calculated as the number of days intervening between the drawing of the blood and the final typing. Here again, the error occurred in an intermediate group—5 days.

Table 2. *Comparison of age of cells in experiment with those of routine samples received by this laboratory*

Age (in days)	Experimental samples	Routine samples
1	0	546
2	0	114
3	54	20
4	657	16
5	263	4
6	195	3
7	57	3
8	123	2
9 or more	68	1

Discussion

If the conditions in the mail were to produce any change in the Rh antigen leading to unreliable results, they should have been revealed by this study. The specimens were treated far more roughly and were much older than the ones we receive routinely. They were shipped over a greater total distance (526 miles) than any ordinary sample received by us. Seventy percent of the specimens had already been in the mail once en route to the State laboratory. In spite of centrifugation, bacterial contamination, removal of serum, age, and exposure to varying degrees of heat and cold, the original results could be reproduced to an extremely high degree of accuracy.

The value of this experiment has been demonstrated in the work of the past year. Out of a total of 27,858 specimens received, 512, or 1.84 percent, were so hemolyzed that previously we would not have attempted the blood grouping. Actually, we were able to type 314 of them, leaving only 0.7 percent of the total to be discarded. With these, repeated attempts to make cell suspensions ended only with a deeply colored supernatant and a little cell debris at the bottom of the tube. No intact cells could be recovered from the clot.

Some caution is necessary in dealing with hemolyzed blood. It is usually impossible to gage the cell suspensions to 2 percent. We make them as heavy as possible in order to get enough uninjured cells for reading the result. If a sufficient number of whole cells can be obtained to get a typical sedimentation pattern in the tube test, the cell debris does not interfere. When a microscopic reading is necessary, the cellular fragments may, at a casual glance, simulate agglutination. Careful viewing is necessary to distinguish between debris and agglutinated cells.

Various preservatives, such as Alsever's solution, ACD mixture, and Rous-Turner solution, have been suggested for keeping the cells intact over long periods of time. These may be of value in isolated instances, but the added cost of providing separate mailing containers reduces their usefulness. Clotted blood is just as effective and can be used for the syphilis test, Rh typing, and titration, while preserved blood is limited to blood grouping.

Summary

A series of cells of known Rh type were sent a distance of 526 miles through the mail. One false negative result was obtained, an error of 0.07 percent. Age of the cells, degree of hemolysis, climatic conditions, and hazards of unknown nature in the mail did not affect the accuracy of the Rh typing.

Incidence of Disease

No health department, State or local, can effectively prevent or control disease without knowledge of when, where and under what conditions cases are occurring

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UNITED STATES

Reports from States for Week Ended June 24, 1950

For the current week in the Nation, reported cases of acute poliomyelitis increased over the number reported the preceding week, 236 to 336. For the corresponding week last year, 409 cases were reported. The States reporting the largest number of cases for the current week were: Texas (107), California (33), Oklahoma (23), New York (17), New Jersey (16), and South Carolina (15).

The cumulative total number of poliomyelitis cases for the calendar year is misleading because the calendar year does not correspond with the disease year. A calendar year covers January through December, while the disease year for poliomyelitis covers March through February. Therefore, the 1950 calendar year includes the declining phase

Comparative Data for Cases of Specified Reportable Diseases: United States

[Numbers after diseases are International List numbers, 1948 revision]

Disease	Total for week ended		5-year median 1945–49	Seasonal low week	Cumulative total since seasonal low week		5-year median 1944–45 through 1948–49	Cumulative total for calendar year		5-year median 1945–49
	June 24, 1950	June 25, 1949			1949–50	1948–49		1950	1949	
Anthrax (062)	1	1	([1])	([1])	([1])	([1])	([1])	20	31	([1])
Diphtheria (055)	44	82	145	27th	[2] 7,280	8,730	13,582	[2] 3,009	3,616	6,016
Acute infectious encephalitis (082)	27	11	9	([1])	([1])	([1])	([1])	344	257	221
Influenza (480–483)	619	572	631	30th	273,998	109,944	179,378	243,468	73,674	135,820
Measles (085)	10,097	10,678	10,678	35th	276,598	611,261	539,754	257,468	560,865	504,808
Meningococcal meningitis (057.0)	73	47	57	37th	3,121	2,786	3,032	2,208	1,944	2,060
Pneumonia (490–493)	987	1,015	-------	([1])	([1])	([1])	-------	54,207	49,170	-------
Acute poliomyelitis (080)	336	409	204	11th	1,662	1,790	1,112	2,796	2,705	1,579
Rocky Mountain spotted fever (104)	19	26	22	([1])	([1])	([1])	([1])	147	206	154
Scarlet fever (050)	631	649	1,053	32d	54,269	78,148	84,707	37,830	55,604	58,021
Smallpox (084)	-------	1	3	35th	44	50	193	24	40	139
Tularemia (059)	20	21	20	([1])	([1])	([1])	([1])	496	612	497
Typhoid and paratyphoid fever (040, 041) [3]	88	74	89	11th	818	783	899	1,328	1,271	1,372
Whooping cough (056)	2,653	1,244	2,052	39th	88,432	36,263	80,315	66,896	26,230	49,049

[1] Not computed. [2] Deduction: North Carolina, week ended June 3, 1 case. [3] Including cases reported as salmonellosis.

of last year's high incidence. On this basis, the cumulative total for the current disease year, or the cumulative total since the seasonal low week (March 18, 1950), is slightly less than the corresponding total for last year, 1,662 cases as compared with 1,790. Corresponding cumulative totals for the higher States showed Texas with 578 cases, California 215, New York 63, Oklahoma 58, Florida 49, Mississippi 46, Iowa 40, and Michigan 38.

The total number of cases of influenza reported for the current week was 619 as compared with 572 for the corresponding period last year. The 5-year (1945–49) median was 631. States reporting the largest number of cases of influenza for the week were: Texas (344), Virginia (91), and Arizona (48).

Reported cases of meningococcal meningitis for the week numbered 73 as compared with 75 last week and 47 for the corresponding week last year. The 5-year (1945–49) median was 57. The largest number of cases was reported by New York with 8.

Reported cases of whooping cough numbered 2,653 for the week as compared with 2,743 last week and 1,244 for the corresponding week last year. The highest corresponding week in the past 5 years was in 1947 when 3,687 cases were reported. The cumulative total since the seasonal low week (October 1, 1949) was 88,432, the second highest total in the last 5 years. The high cumulative total during this 5-year period was 1947 when 98,505 cases were reported. The low cumulative total number of cases of whooping cough was 36,263 for 1949.

The total number of cases of infectious encephalitis reported for the week was 27 as compared with 22 last week, 11 for the corresponding week last year, and 9 for the 5-year (1945–49) median. The cumulative total for 25 weeks of this calendar year was 344 which was higher than any corresponding total the past 5 years. The corresponding 5-year median was 221 cases.

Deaths During Week Ended June 24, 1950

	Week ended June 24, 1950	*Corresponding week, 1949*
Data for 94 large cities of the United States:		
Total deaths	8, 730	8, 877
Median for 3 prior years	8, 684	----------
Total deaths, first 25 weeks of year	240, 031	237, 797
Deaths under 1 year of age	574	583
Median for 3 prior years	612	----------
Deaths under 1 year of age, first 25 weeks of year	15, 518	16, 167
Data from industrial insurance companies:		
Policies in force	69, 748, 425	70, 389, 046
Number of death claims	12, 986	12, 166
Death claims per 1,000 policies in force, annual rate	9. 7	9. 0
Death claims per 1,000 policies, first 25 weeks of year, annual rate	9. 8	9. 5

Reported Cases of Selected Communicable Diseases: United States, Week Ended June 24, 1950

[Numbers under diseases are International List Numbers, 1948 revision]

Area	Diphtheria (055)	Encephalitis, infectious (082)	Influenza (480–483)	Measles (085)	Meningitis, meningococcal (057.0)	Pneumonia (490–493)	Poliomyelitis (080)
United States	44	27	619	10,097	73	987	336
New England	**3**	**1**	**1**	**1,374**	**2**	**26**	**5**
Maine			1	9		2	1
New Hampshire				37			
Vermont				1			
Massachusetts	3	1		909	1		3
Rhode Island				2		6	
Connecticut				416	1	18	1
Middle Atlantic	**8**	**9**	**2**	**3,099**	**16**	**265**	**36**
New York	3	5	[1] 2	1,163	8	188	17
New Jersey	1	3		1,196	2	23	16
Pennsylvania	4	1		740	6	54	3
East North Central	**8**	**6**	**12**	**2,939**	**11**	**143**	**21**
Ohio	3		1	496	4	21	5
Indiana	2	1		141		3	3
Illinois		3	2	941	4	76	5
Michigan	2	2	1	573	2	35	4
Wisconsin	1		8	788	1	8	4
West North Central	**3**	**3**	**12**	**475**	**8**	**81**	**17**
Minnesota	1	1	5	60	1	24	1
Iowa	1			177	2	1	9
Missouri		1	1	66	2	13	5
North Dakota			1	8	1	26	
South Dakota		1		43			
Nebraska			5	88	1	2	2
Kansas	1			33	1	15	
South Atlantic	**5**	**1**	**115**	**448**	**9**	**124**	**38**
Delaware				20			
Maryland			4	61		30	
District of Columbia			2	22		23	1
Virginia	1		91	72	2	29	3
West Virginia	1		1	43	1	10	4
North Carolina		1		54	3		5
South Carolina			12	23		12	15
Georgia			5	21	2	7	1
Florida	3			132	1	13	9
East South Central	**7**	**1**	**10**	**244**	**8**	**69**	**21**
Kentucky	3		1	130	2	9	5
Tennessee		1	6	78	1		2
Alabama	2		2	32	5	43	5
Mississippi	2		1	4		17	9
West South Central	**7**	**3**	**375**	**327**	**15**	**202**	**140**
Arkansas		1	13	32	2	4	8
Louisiana				11	5	16	2
Oklahoma			18	19	2	10	23
Texas	7	2	344	265	6	172	107
Mountain	**1**	**1**	**79**	**566**	**1**	**44**	**19**
Montana			10	34		2	
Idaho			4	64			1
Wyoming				20			6
Colorado			15	252	1	16	3
New Mexico			2	20		16	2
Arizona			48	19		7	6
Utah	1	1		157		3	1
Nevada							
Pacific	**2**	**2**	**13**	**625**	**3**	**33**	**39**
Washington				41			1
Oregon			5	10	1	13	5
California	2	2	8	574	2	20	33
Alaska						1	
Hawaii				5			2

[1] New York City only.

Anthrax: New Jersey, 1 case.

Reported Cases of Selected Communicable Diseases: United States, Week Ended June 24, 1950—Continued

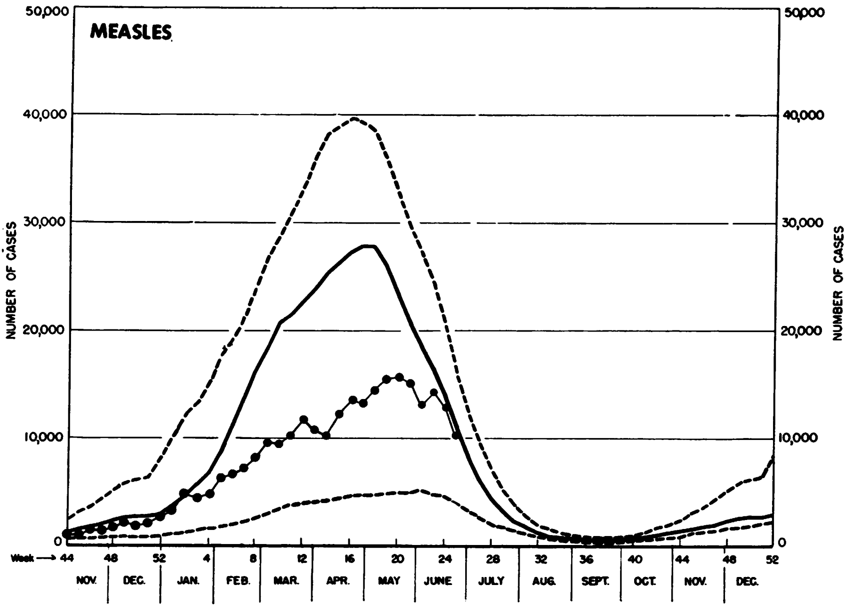
[Numbers under diseases are International List Numbers, 1948 revision]

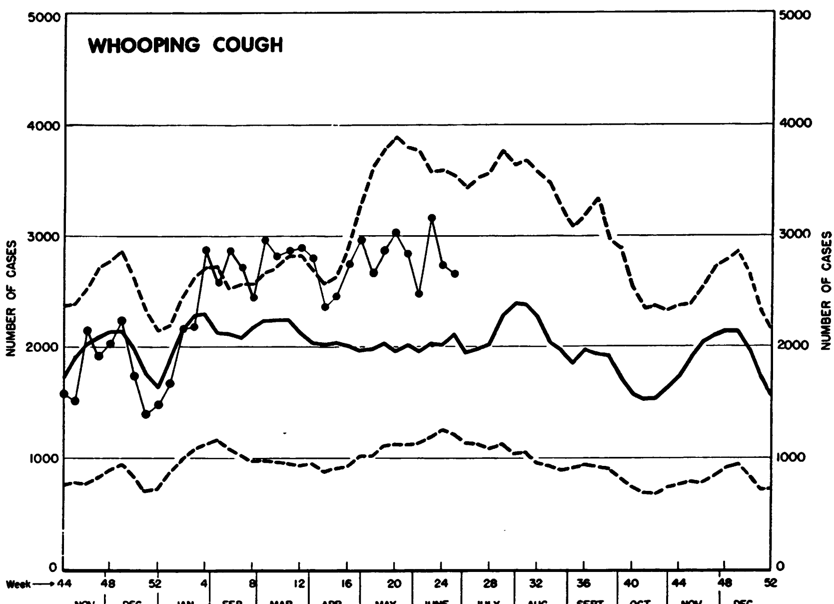
Area	Rocky Mountain spotted fever (104)	Scarlet fever (050)	Small-pox (084)	Tularemia (059)	Typhoid and paratyphoid fever [1] (040, 041)	Whooping cough (056)	Rabies in animals
United States	19	631		20	88	2,653	130
New England		**101**			**2**	**241**	
Maine		1				22	
New Hampshire		2				1	
Vermont						20	
Massachusetts		87			1	95	
Rhode Island		2				45	
Connecticut		9			1	58	
Middle Atlantic		**135**			**6**	**326**	**23**
New York		[2] 83			3	148	22
New Jersey		11			1	107	
Pennsylvania		41			2	71	1
East North Central	**2**	**138**		**1**	**8**	**458**	**33**
Ohio		44			1	89	4
Indiana	1	1				20	11
Illinois	1	16		1	5	76	2
Michigan		57			2	172	15
Wisconsin		20				101	1
West North Central		**25**		**2**		**155**	**16**
Minnesota		8				21	
Iowa		3				36	16
Missouri		7		2		33	
North Dakota		1				24	
South Dakota		1				3	
Nebraska		4				1	
Kansas		1				37	
South Atlantic	**11**	**43**		**3**	**18**	**423**	**12**
Delaware		2				7	
Maryland	3	11			1	42	
District of Columbia		1				3	
Virginia	4	12				100	2
West Virginia	1	5			8	85	6
North Carolina	1	6			1	135	
South Carolina	2	1		1	4	16	2
Georgia		4		1	4	11	2
Florida		1		1		24	
East South Central	**3**	**32**			**8**	**107**	**20**
Kentucky	1	12			1	21	11
Tennessee		11			4	49	5
Alabama	1	6			1	28	3
Mississippi	1	3			2	9	1
West South Central	**1**	**29**		**11**	**26**	**508**	**25**
Arkansas	1			8	3	34	2
Louisiana		2			5	12	
Oklahoma		12		1	4	36	4
Texas		15		2	14	426	19
Mountain	**2**	**16**		**3**	**1**	**185**	**1**
Montana	2	1		2		33	
Idaho		1				18	
Wyoming		1					
Colorado		3				19	
New Mexico		5			1	43	
Arizona		3				53	1
Utah		2		1		19	
Nevada							
Pacific		**112**			**19**	**250**	
Washington		11				76	
Oregon		4				37	
California		97			19	137	
Alaska						30	
Hawaii		1				1	

[1] Including cases reported as salmonellosis. [2] Including cases reported as streptococcal sore throat.

Communicable Disease Charts

All reporting States, November 1949 through June 24, 1950





The upper and lower broken lines represent the highest and lowest figures recorded for the corresponding weeks in the 5 preceding years. The solid line is a median figure for the 5 preceding years. All three lines have been smoothed by a 3-week moving average. The dots represent numbers of cases reported for the weeks, 1949–50.

FOREIGN REPORTS

CANADA

Reported Cases of Certain Diseases—Week Ended June 10, 1950

Disease	Newfoundland	Prince Edward Island	Nova Scotia	New Brunswick	Quebec	Ontario	Manitoba	Saskatchewan	Alberta	British Columbia	Total
Brucellosis					1	1					2
Chickenpox			20		106	213	20	6	59	90	514
Diphtheria				1	1				1		3
Dysentery, bacillary										1	1
Encephalitis, infectious						1					1
German measles			31			1, 388	1	84	99	359	1, 962
Influenza			25			1					26
Measles			3		482	345	43	15	17	199	1, 104
Meningitis, meningococcal						3		1			4
Mumps			56		150	267	11	57	93	129	763
Poliomyelitis					1	1				3	5
Scarlet fever			1		49	34	2	5	33	5	129
Tuberculosis (all forms)	17		7	6	90	33	8	8	21	38	228
Typhoid and paratyphoid fever					1	1					2
Venereal diseases:											
Gonorrhea	3		5	13	116	46	12	17	31	47	290
Syphilis			8	2	57	26	3	12	3	10	121
Other forms										1	1
Whooping cough	2		2		65	49	1	3		98	220

JAPAN

Influenza. During the epidemic of influenza which began in Japan early in December 1949, and ended the latter part of April 1950, a total of 15,904 cases was reported. The peak incidence occurred during the week ended February 11, when 2,746 cases were reported.

MADAGASCAR

Reported Cases of Certain Diseases and Deaths—April 1950

Disease	Aliens		Natives	
	Cases	Deaths	Cases	Deaths
Beriberi			1	
Bilharziasis	1		49	
Diphtheria	4	3	5	2
Dysentery:				
Amebic	17		254	23
Bacillary			59	4
Erysipelas			18	1
Influenza	20		3, 308	52
Leprosy			40	
Malaria	277		42, 033	214
Measles			73	
Meningitis, meningococcal			1	
Mumps	1		322	
Plague			6	5
Pneumonia (all forms)	6		841	146
Puerperal infection			2	
Tuberculosis, pulmonary	12	2	103	11
Typhoid fever	1		10	5
Whooping cough	16	5	351	4

REPORTS OF CHOLERA, PLAGUE, SMALLPOX, TYPHUS FEVER, AND YELLOW FEVER RECEIVED DURING THE CURRENT WEEK

The following reports include only items of unusual incidence or special interest and the occurrence of these diseases, except yellow fever, in localities which had not recently reported cases. All reports of yellow fever are published currently. A table showing the accumulated figures for these diseases for the year to date is published in the PUBLIC HEALTH REPORTS for the last Friday in each month.

Cholera

Burma. During the week ended June 10, 1950, one fatal case of cholera was reported in Akyab, and one fatal case was reported in Bassein.

India. During the week ended June 10, 1950, 318 cases of cholera were reported in Calcutta, and 283 cases were reported during the week ended June 17. One case was reported in Bombay during the week ended June 10. This is the first indigenous case reported during the current year.

Pakistan. During the week ended June 10, 1950, 10 cases of cholera, with 6 deaths were reported in Chittagong.

Plague

China. During the month of March 1950, 37 cases of plague, with 14 deaths, were reported in the Districts of Fukien Province. Nine deaths were also reported on Kinmen Island March 21–31. This island is located near Amoy.

Peru. During the month of April 1950, one case of plague was reported in Huacho, Chancay Province, Lima Department.

Smallpox

Cameroon (French). Smallpox has been reported in French Cameroon as follows: May 1–10, 1950, 30 cases, 9 deaths; May 11–20, 11 cases, 1 death.

Chile. During the period May 23–June 3, 1950, 318 cases of smallpox were reported in Chile. The highest reported incidence occurred in Malleco Province, where 183 cases were recorded.

Greece. Smallpox has been reported in Greece as follows: During the period June 6–12, 1950, 8 cases in Attica Department; on June 9, one case in Athens; on June 19, one fatal case in the port of Xylokastron; on June 20, one case in Piraeus.

Indonesia (Java). Smallpox has been reported in Surabaya, as follows: May 1–31, 1950, 605 cases, 174 deaths; week ended June 3, 151 cases; week ended June 10, 172 cases.

Pakistan. During the period May 6–27, 1950, 113 cases of smallpox, with 78 deaths, were reported in Dacca.

Typhus Fever

Afghanistan. During the period May 1–31, 1950, 248 cases of typhus fever were reported.

Ecuador. During the month of April 1950, 18 cases of typhus fever (including 5 cases in Quito) were reported in Ecuador.

Egypt. During the week ended June 10, 1950, 4 cases of typhus fever were reported in Cairo.

India. Typhus fever has been reported in Kashmir State, as follows: Week ended April 15, 1950, 30 cases, 14 deaths; week ended April 22, 8 cases, 5 deaths; week ended May 6, 31 cases, 1 death.

Jamaica. During the week ended June 3, 1950, 5 cases of murine typhus fever were reported in Kingston.

Japan. The outbreak of typhus fever which began in Tokyo during the week ended January 14, 1950, and later spread to Yokohama, was reported to have reached its peak in Tokyo during the week ended February 11, and in Yokohama during the week ended February 18. The total number of cases reported in all Japan for the period January 1–April 15, 1950, was 731 (49 deaths). Of these, 222 cases, 19 deaths, were reported in Tokyo, and 423 cases, 23 deaths, in Yokohama.

Republic of Korea. During the month of May 1950, 57 cases of typhus fever (murine type) were reported in Seoul.

Yellow Fever

Peru. One death from yellow fever in San Ramon, Junin Department, was reported confirmed early in June 1950. The specific dates of onset and confirmation were not stated.

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Plague Infection in Kittitas County, Wash.

Under date of June 22, 1950, plague infection was reported proved in a specimen of 42 fleas as follows: *Megabothris abantis* 20, *Meringis shannoni* 11, *Thrassis gladiolis johnsoni* 7, and *Malaræus telchinum* 4. This specimen was taken from 8 sagebrush voles, *Lagurus curtatus*, trapped June 7, 1950, 18 miles east of Ellensburg on United States Highway 10, Kittitas County, Wash.

Tularemia Infection in a Tick, Valencia County, N. Mex.

Under date of June 22, 1950, tularemia infection was reported proved in a specimen of one tick taken from a cottontail rabbit, *Sylvilagus auduboni*, shot June 7, near El Morro National Monument, Valencia County, N. Mex.