LABORATORY REPORT No.301

INDIRECT HEMAGGLUTINATION TEST FOR MYCOPLASMA PNEUMONIAE EMPLOYING A COMMERCIALLY AVAILABLE ANTIGEN AND ADAPTED TO THE MICROTITER TECHNIC

1 DECEMBER, 1966

US ARMY MEDICAL RESEARCH AND NUTRITION LABORATORY

FITZSIMONS GENERAL HOSPITAL
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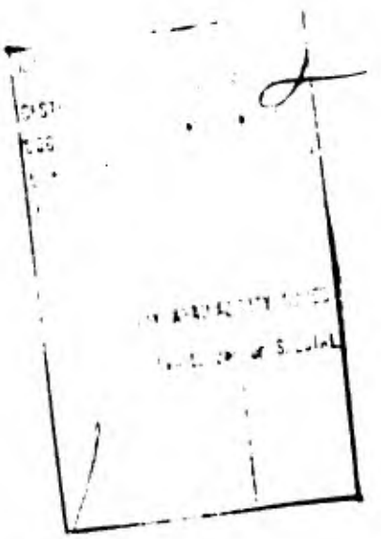

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REPORT NO. 301

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Project Number: 3A025601A822 Military Internal Medicine
Task No.: 01
Work Unit No.: 066 Miscellaneous Microbiological Clinical Research and Support

INDIRECT HEMAGGLUTINATION TEST FOR MYCOPLASMA PNEUMONIAE EMPLOYING A COMMERCIALLY AVAILABLE ANTIGEN AND ADAPTED TO THE MICROTITER TECHNIC

By

Avrum B. Organick
Abraham Resnick

U. S. Army Medical Research and Nutrition Laboratory

ABSTRACT

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Project No. 3A025601A822 Military Internal Medicine
Task No. 01
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Two Mycoplasma pneumoniae antigens were compared employing an indirect hemagglutination method modified to a microtiter technic.

Sonic treatment of the antigen immediately prior to sensitizing erythrocytes is an important step in the procedure.

The IHA test must be performed on a larger number of sera in order to evaluate its usefulness.

Other commercial sources of antigen for sensitizing erythrocytes should be investigated.

BODY OF REPORT

WORK UNIT NUMBER 066 Miscellaneous Microbiological Clinical Research and Support

INTRODUCTION:

An indirect hemagglutination test for the diagnosis of Mycoplasma pneumoniae infections was described by Dowdle and Robinson (1) based upon Stavitsky's technic of employing tanned sheep erythrocytes sensitized with a specific antigen (2). Chanock, in a recent review (3), has pointed out that the indirect hemagglutination (IHA) test rises early in M. pneumoniae infections and this may represent an advantage in earlier detection. The IHA test, in addition, possesses a technical advantage; namely, that sensitized, tanned erythrocytes may be frozen in dextrose and lactose and preserved at temperatures of -70 C making available a prepared, standardized antigen when needed (1, 4). It is the object of this report to describe efforts to use a readily available commercial M. pneumoniae (CF) antigen for sensitizing erythrocytes and to adapt the IHA test to the Microtiter* technic.

METHODS:

Antigens. Two M. pneumoniae antigens were employed in these studies: (1) a concentrated, "raw" antigen prepared by the harvesting of M. pneumoniae from a mycoplasma broth culture of the M-52 strain of M. pneumoniae, resusperding after washing in 1/200th the original volume, and disrupting the cells by subjecting them to 3 alternate freeze-thaw cycles (hereinafter referred to as 'M' antigen), and, (2) an undiluted, commercially prepared M. pneumoniae antigen** employed for the complement fixation test (and designated as 'C' antigen).

Sera. Sera from patients with M. pneumoniae infections from the Milwaukee County Hospital and from Fitzsimons General Hospital and which had known CF titers for M. pneumoniae as well as the serum from a patient with a negative M. pneumoniae CF titer were employed.

Indirect Hemagglutination (IHA) Test. The general procedure described by Dowdle and Robinson (1) was employed. Phosphate buffered saline (PBS) was originally made up as described by Stavitsky (2) but in later experiments was prepared according to the method of the Media Preparation Section of the National Institutes of Health as follows:

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* Cooke Engineering Co., Alexandria, Virginia

* The use of commercial trade names is for the purpose of identification only and does not constitute an endorsement by the Department of Defense.

** Eaton Agent C. F. Antigen, Baltimore Biological Laboratories, Baltimore, Md.

Saline, pH 6.4: NaCl, 8.5 Gm; H_2O 900 ml; M/15 Na_2HPO_4, 26.7 ml; M/15 KH_2PO_4 73.3 ml.

Saline, pH 7.2: NaCl 8.5 Gm; H_2O 900 ml; M/15 Na_2HPO_4, 72.0 ml; M/15 KH_2PO_4, 28.0 ml.

The pH was checked with a pH meter and adjusted when necessary with either M/15 Na_2HPO_4 or KH_2PO_4. No difference in the appearance of the red cell suspensions was noted when buffers were prepared by either method.

PBS of pH 7.2 was used except where noted. The final suspensions of sensitized cells and all the test serum dilutions were made in heat-inactivated normal rabbit serum which had been absorbed with concentrated sheep erythrocytes and diluted 1:150 in PBS.

Sheep erythrocytes in Alsever's solution were washed 3 times with PBS, adjusted to a final concentration of 2.5%, and mixed with an equal volume of 1:20,000 tannic acid (except where 1:10,000 was used) in PBS. (Fisher Certified Reagent grade tannic acid was employed to prepare a 1% stock solution; 0.5 ml of the 1% stock solution was diluted to 100 ml. in PBS to make a fresh preparation of 1:20,000 for each use.) After 10 minutes' incubation at 37°C the cells were centrifuged, washed once in PBS, and resuspended to 2.5% in PBS at pH 6.4.

Sensitization of the tanned erythrocytes was carried out as follows: to one volume of antigen was added one volume of the 2.5% suspension of tannic acid-treated cells and four volumes of PBS, pH 6.4, and the mixture incubated for 30 minutes at room temperature. The cells were washed twice in rabbit serum diluent and adjusted to a 0.5% suspension in the same diluent.

All sera to be tested were inactivated at 56°C for 30 minutes, diluted 1:10 in rabbit serum diluent, and adsorbed at 4°C for 30 minutes using 0.1 ml of concentrated erythrocytes per ml of diluted serum.

In the macro test (employing 6 ml serologic tubes) the treated sera were titrated in 2-fold dilutions using 0.2 ml rabbit serum diluent and an equal volume of a 0.5% sensitized erythrocytes was added.

In the Microtiter test 0.025 ml of rabbit serum diluent was delivered with the 0.025 ml calibrated delivery pipette to each cup except the first in each line of cups. 'V' and, where described, the 'U' Microtiter plates were used. 0.025 ml of each treated and diluted (1:10) serum was delivered to the first two cups in two lines, one line for the test employing the sensitized erythrocytes and one line for a control employing a 0.5% solution of tanned but unsensitized cells. Two additional controls were employed in each experiment: (1) a diluent

control consisting of a single cup to which 0.025 ml of rabbit serum diluent and 0.025 ml of sensitized erythrocytes were added, and, (2) a complete titration of a known positive serum. Serial 2-fold dilution of the sera were performed beginning with the second cup in each line and employing calibrated Microtiter loops. 0.025 ml of sensitized erythrocytes were added to each cup in the test line, and 0.025 ml of tanned but unsensitized erythrocytes were added in the tanned RBC control line.

The Microtiter plates were sealed and the test incubated at 37°C for 3 hours. At the end of this period the plates and tubes were centrifuged for 2 minutes at 400 rpm (employing International head #276), placed in the refrigerator overnight and read the following morning. The reciprocal of the highest initial dilution yielding a clearly positive agglutination of the sensitized erythrocytes was considered the end-point.

Results

In an initial experiment the raw 'M' antigen was employed in the IHA test using 11 sera in Microtiter 'U' plates and two sera in serologic tubes. No readable hemagglutination was obtained. It was clear that the freeze-thaw cycles employed were insufficient to disrupt the mycoplasma cells in the antigen preparation 'M'. Accordingly, the remaining 3 ml of 'M' antigen was diluted to 10 ml, placed in a reaction vessel, sonically disrupted for 10 minutes with the probe of a Bronson "sonifier" (Model LS 75), left hand knob setting at '3' and right hand variable knob set to produce a visible, active foaming, and this antigen was employed in a repeat IHA test. Because it was of concern that the remaining quantity of 'M' antigen might be insufficient for any long use, antigen from a commercial source, originally prepared for use in the complement fixation test (Eaton Agent C. F. Antigen, Baltimore Biological Laboratories) was employed in a duplicate series in the IHA test along with the sonically disrupted 'M' antigen. A further object of the repeat experiment was to compare the readings obtained with the 'U' and 'V' Microtiter plates, and to compare these in turn with readings of the gross, serologic tube test.

Reading of Reaction. Distinct readable results were obtained with the second experiment. The criteria employed for reading the reactions on both the 'U' and 'V' Microtiter plates will be described. The criteria for reading the results of the gross serologic tube test are those of Stavitsky (2).

Microtiter 'U' Plate:

Negative	Small, thick, discrete ring or dot.
+ + + +	Little or no ring visible. If present, indistinct, irregular, and of large circumference. Central mat present with or without visible clumps and/or clear areas.
+ + +	Ring present but thin and of large circumference.
+ +	Distinct ring but of larger circumference than control.
+ to ±	Varying degrees of smaller, thicker, more distinct ring - difficult to distinguish from the control.

Microtiter 'V' Plate:

Negative	Strong, bright, central, red button.
+ + + +	Complete failure of formation of button.
+ + +	Button formation but small, irregular, light colored, and with surrounding clumps.
+ +	But on better formed than above but still distinctly smaller than control.
+ to ±	Varying degrees of bright distinct central red button formation difficult to distinguish from the control.

The various reactions are illustrated in the photographs (Figs. 1 - 2). Table 1 lists the sera employed and the CF titers of those sera. Table 2 summarizes the results of the second experiment and permits comparison of the sonically disrupted 'M' antigen with the 'C' antigen, of Microtiter 'U' and 'V' plates, and of these, in turn, with the gross serologic tube test. It was clear that readings employing Microtiter plates gave titers higher than the serologic tubes, and that the 'M' antigen was more sensitive than the 'C' antigen. Because of the sharpness of the central red button in the control cups in the 'V' Microtiter plates and the increased ease with which the reactions could be read, the 'V' Microtiter plates were considered superior to the 'U' plates.

Several factors were considered in an attempt to explain the failure of the 'C' antigen to produce as sensitive an erythrocyte-antigen combination as the 'M' antigen-sensitized cells. The first factor was the possibility that the 1:20,000

solution of tannic acid employed to tan the red cells might be too weak to provide effective binding sites for the 'C' antigen. The second factor was the possibility that the 'C' antigen might be improved as a sensitizer of erythrocytes if it too were sonically disrupted, since sonification improved the effectiveness of the cruder 'M' antigen. Accordingly an additional experiment was designed to test these two possibilities. Erythrocytes were prepared for sensitization by antigen which had been tanned with either a 1:20,000 or a 1:10,000 solution of tannic acid. To lines of serial dilutions of the test sera in Microtiter 'V' plates were added sensitized erythrocytes which received prior tanning with the 1:20,000 and with the 1:10,000 tannic acid solution, each test accompanied by its own control of unsensitized but correspondingly tanned erythrocytes. Two antigens were compared: the 'M' antigen, thawed after storage in the freezer for 3 days and not subjected again to sonic treatment and the 'C' antigen, sonically treated for 10 minutes in the same manner as had been employed several days earlier with the 'M' antigen.

The effect of the increased concentration of tannic acid upon the erythrocytes was apparent at once. A considerable degree of agglutination of the unsensitized control cells which had been tanned with the 1:10,000 solution of tannic acid rendered impossible any reading of the corresponding hemagglutination test. Treatment of erythrocytes with 1:20,000 tannic acid, as had been the case with the previous experiments, produced excellent, sharp, negative controls in each case.

The results of the hemagglutination test in which 'M' and sonically treated 'C' antigens were employed to sensitize 1:20,000 tannic acid-treated cells are summarized in Table 3. The table also includes the results of the Microtiter 'V' plate readings of the previous experiment performed several days earlier and the CF titers of the sera. Some degree of improvement in the sensitivity of the 'C' antigen following sonic treatment is noted in serums #8 and #11. There was an apparent decrease in sensitivity of the HA test for serum #10. More striking than the effects of sonic treatment of the 'C' antigen, however, was the marked loss of activity of the 'M' antigen compared with its activity several days earlier.

Discussion

The studies described in this report emphasize that readable results can be obtained with the IHA test described by Dowdle and Robinson when applied to the Microtiter technic and when a readily available, commercial antigen is employed. Sonic treatment of the antigen before use is probably a technical point of great importance. The number of sera examined in these studies is too small to permit any evaluation on the basis of these data of the superiority of the IHA test, if any, over the complement fixation test. Agreement between the degrees of positivity of the IHA and the CF test was not always

present. In serum #9, for example, although the complement fixation test was positive in a titer of 1:32, the IHA test was consistently negative. The explanation for this may lie in the fact that the serum may represent a late convalescent specimen which still contained detectable CF antibodies, but in which hemagglutinating antibodies had already disappeared. The answers to these problems can only come from careful studies which correlate the clinical course and the serological events in large numbers of patients.

One disadvantage of the IHA test is that it involves at least one additional step when compared with the CF test; namely, that each serum must be adsorbed with erythrocytes to remove naturally occurring anti-sheep cell agglutinins and hemolysins from patients' sera.

These studies indicate that a freshly sonified broth culture concentrate of M. pneumoniae may be superior to the commercial BBL CF antigen. The advantages of having an available antigen of fairly uniform potency from commercial sources may outweight the disadvantage of a slight decrease in potency compared with a broth concentrate produced in small batches. Other commercial sources of M. pneumoniae antigen may be more potent than the BBL antigen for the IHA test.

Summary and Conclusions

1. The indirect hemagglutination (IHA) test for Mycoplasma pneumoniae infections can be performed employing a readily available antigen for sensitizing tanned red blood cells, and this test can be adapted to the Microtiter technic.

2. Sonic treatment of the antigen immediately prior to sensitizing erythrocytes is an important step in the procedure.

3. The IHA test must be performed in a larger number of sera in this laboratory in order to evaluate its usefulness.

4. Other commercial sources of antigens for sensitizing erythrocytes should be investigated.

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(1). Dowdle, W. R. and Robinson, R. Q. An indirect hemagglutination test for the diagnosis of Mycoplasma pneumoniae infections. Proc. Soc. Exper. Biol. and Med., 116:947-950, 1964.

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(4). Hubert, E. G., Kalmanson, G. M., and Guze, L. B. Preservation of antigen-coated sheep erythrocytes by freezing for use in indirect hemagglutination procedure. J. Bacteriol. 86:569-572, 1963.

NOTE: This work was done while the authors were temporarily assigned from the 452nd General Hospital, USAR to the USAMRNL (MI), Fitzsimons General Hospital, Denver, Colorado 80240 during June 1966.

As of the date of this report, Dr. Organick's current address is:

Avrum B. Organick, M. D.
(Major, MC, USAR)
Assistant Professor of Medicine
Marquette University School of Medicine
Milwaukee County General Hospital
8700 West Wisconsin Avenue
Milwaukee, Wisconsin 53226

TABLE I

SERA

EMPLOYED IN HEMAGGLUTINATION TEST FOR *MYCOPLASMA PNEUMONIAE*

AND THEIR COMPLEMENT FIXATION TITERS

NUMBER	NAME	SOURCE	CF TITER
4	Grieshop	MCH	NEG
5	Kult	MCH	1:128
8	Williams (7 June 66)	FGH	-
9	Baber	FGH	1:32
10	Kronkwright	FGH	1:128
11	Dapper	FGH	1:128
12	Williams (13 June 66)	FGH	-

TABLE 2

MYCOPLASMA PNEUMONIAE HEMAGGLUTINATION TEST:
COMPARISON OF RECIPROCAL TITERS WITH 'M' AND 'C'
ANTIGENS EMPLOYING 'U' AND 'V' MICROTITER PLATES AND
SEROLOGIC TUBES

SERUM #	'M' ANTIGEN			'C' ANTIGEN		
	'U' PLATE	'V' PLATE	SEROLOGIC TUBE	'U' PLATE	'V' PLATE	SEROLOGIC TUBE
4	NEG	NEG	NEG	NEG	NEG	NEG
5	320	320	-	NEG	NEG	-
8	320	320	-	160	NEG	-
9	NEG	-	NEG	NEG	-	-
10	1280	1280	-	160	1280	-
11	1280	> 1280	320	1280	1280	NEG

TABLE 3

COMPARISON OF MYCOPLASMA PNEUMONIAE HEMAGGLUTINATION TEST EMPLOYING 'M' AND 'C' ANTIGEN ON DIFFERENT DAYS AND SHOWING CORRESPONDING CF TITERS

SERUM #	11 June		13 June		CF TITER
	'M'	'C'	'M'	'C'	
4	NEG	NEG	NEG	NEG	NEG
5	320	NEG	-	-	128
8	320	NEG	NEG	160	-
9	-	-	NEG	NEG	32
10	1280	1280	NEG	320	128
11	> 1280	1280	NEG	2560	128
12	-	-	NEG	320	-

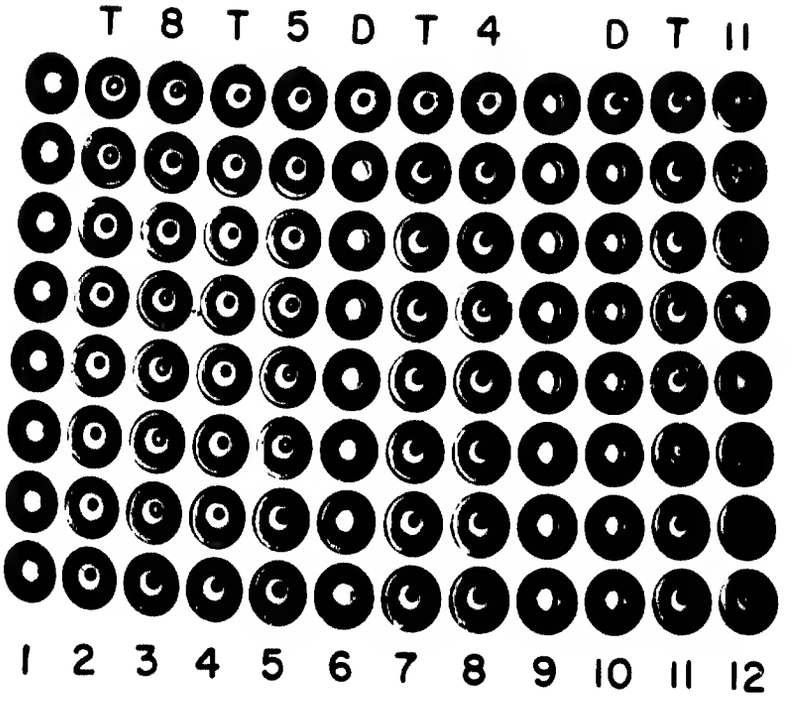


Figure 1. Hemagglutination test employing Microtiter 'U' plate. The first two rows on the right (marked 11 and 12 on the bottom of the figure, and, faintly visible, marked "T", for tanned red cell control, and "11", for the number of the serum, on a tape along the top of the Microtiter plate) represent a positive test. The readings in the test row from top to bottom are as follows: 2+, 2+, 3+, 4+, 4+, 3+, 2+, 2+. The hemagglutination test for this serum was therefore read as 1:1280. Note the prozone phenomenon. The agglutination visible in the sixth tube in the control line was due to inadvertent introduction of sensitized cells into this cup. The remaining agglutination reactions of the plate are negative.

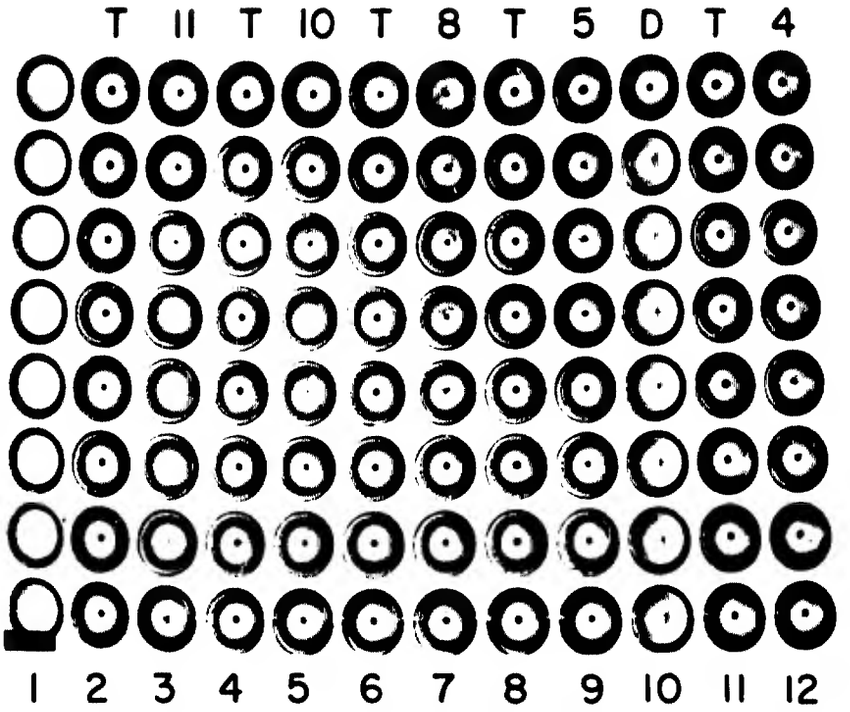
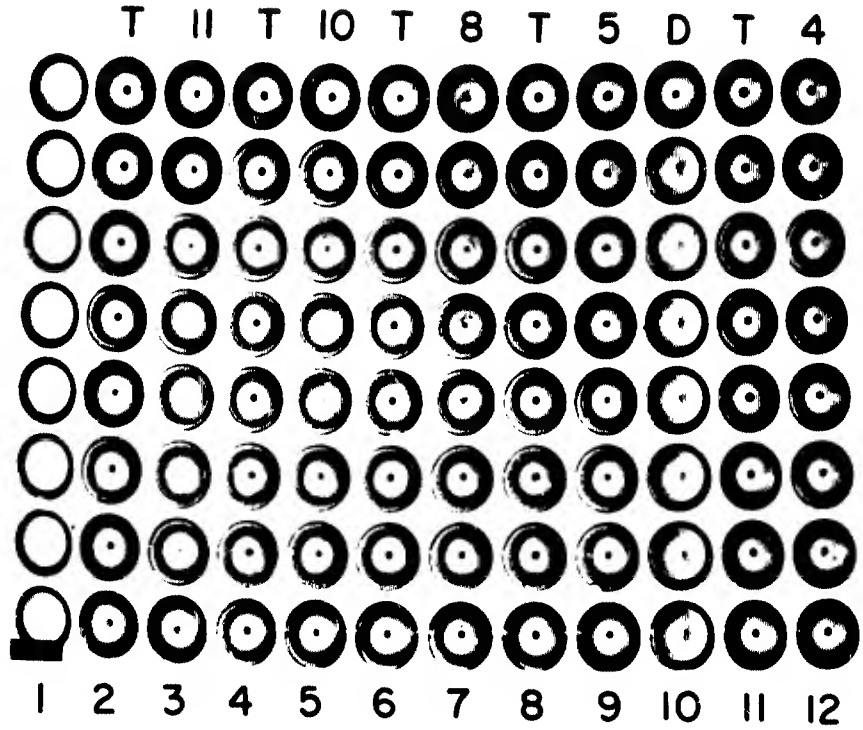


Figure 2. Hemagglutination test employing Microtiter 'V' plate. The second and third, fourth and fifth, sixth and seventh, and eighth and ninth rows from the left (representing, as indicated on the tape above, serums number 11, 10, 8, and 5, each with its companion tanned red cell control) represent positive hemagglutination reactions. The readings from top to bottom are as follows: serum #11 (row 3), 2+, 2+, 3+, 4+, 4+, 4+, 4+, 3+ for a reading of >1:1280; serum #10 (row 5), neg, 1+, 3+, 4+, 4+, 2+, 2+, 1+ for a reading of 1:640; serum #8 (row 7), 2+, 2+, 3+, 3+, 3+, 2+, 1+, 1+ for a reading of 1:320; serum #5 (row 9), neg, 2+, 3+, 3+, 2+, 2+, 1+, 1+, for a reading of 1:320. Serum #4 (row 12) illustrates a negative hemagglutination reaction.



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13 ABSTRACT

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