

AGGLUTINATION OF *TREPONEMA PALLIDUM* IN HUMAN SYPHILIS.

By JOHN A. KOLMER, M.D., STUART BROADWELL, JR., AND TOITSU MATSUNAMI, M.D.

(From the McManes Laboratory of Experimental Pathology of the University of Pennsylvania, Philadelphia.)

(Received for publication, June 7, 1916.)

In 1913 one of us (1) first described agglutination of a pure culture of *Treponema pallidum* by the sera of rabbits injected with a living and heat-killed culture.¹ Normal rabbit sera were found not to agglutinate this culture in dilutions as low as 1:20 (lower dilutions were not used), while the sera of immunized animals were found capable of agglutinating the culture in dilutions as high as 1:1,280. The agglutinating power of the sera of nineteen individuals in various stages of syphilis and of ten non-syphilitic individuals was studied in dilutions ranging from 1:20 to 1:640, but with negative results, due in large part, as we now believe, to the fact that lower dilutions of serum were not employed.

Previous to this, attempts had been made by Hoffmann and von Prowazek (2), Herxheimer and Löser (3), Hoffmann (4), Brønnum and Ellermann (5), Babes and Panea (6), Metchnikoff and Roux (7), Landsteiner and Mucha (8, 9), Zabolotny and Maslakowetz (10), and Uhlenhuth and Mulzer (11) to determine the presence of agglutinins for *Treponema pallidum* in the sera from human and experimental syphilis according to the grouping of the spirochetes in the tissues and with extracts and emulsions of tissues rich in the microparasites. While the presence of an agglutinin was suspected, technical difficulties, owing to the emulsion employed in the absence of pure cultures, did not permit the deduction of any positive conclusions.

In 1913, Nakano (12) found agglutinins for a culture of *Treponema pallidum* in the sera of rabbits injected intravenously with a heat-killed culture, that yielded well marked agglutination in dilutions from 1:10 to 1:70. Kissmeyer (13) working with a pure culture reported that normal human serum may agglutinate *Treponema pallidum* in dilutions as high as 1:50; he also tested the sera of 59 individuals in the various stages of syphilis and reported positive results in dilutions of 1:100 and higher in about 40 to 60 per cent of cases in the primary, secondary, and tertiary stages, including congenital syphilis, while the sera of immunized rabbits were reported as yielding well marked agglutination in dilutions

¹ The culture was obtained from Dr. Hideyo Noguchi of The Rockefeller Institute for Medical Research.

ranging from 1:2,000 to 1:200,000. Zinsser and Hopkins (14) have found that normal rabbit serum may agglutinate *Treponema pallidum* in dilutions of 1:10 and lower, and the sera of immunized animals as high as 1:2,000. They also stated that "normal human sera will agglutinate similar *pallidum* emulsions, as will the sera of certain syphilitic patients with positive Wassermann reactions," but gave no details of this portion of their work. In a second paper Zinsser, Hopkins, and McBurney (15) present important evidence indicating that the immune agglutinins in the sera of immunized rabbits and sheep failed to cause agglutination of virulent *pallida* from human lesions.

In this communication we desire to give the results of a study of agglutination of a pure culture of *Treponema pallidum* by normal human sera, the sera of persons in the various stages of syphilis, and the sera of non-syphilitic persons suffering with various other diseases.

Method.

Sera.—The sera of 12 persons known not to be syphilitic and with negative Wassermann reactions were studied; also the sera of 5 persons late in the primary, and 12 in the secondary stages of syphilis; 22 from patients with paresis; 12 from patients with positive Wassermann or luetin reactions and regarded as being tertiary or latent syphilitic; and 8 from persons with negative Wassermann reactions and denying syphilitic infection, but suffering with various other infections at the time when these tests were made.

Culture.—All the tests here reported were conducted with Zinsser's Strain A culture of *Treponema pallidum*. This culture was received growing under paraffin oil in ascitic broth over an egg medium and has been successfully grown in large tubes of egg medium covered with ascitic broth and paraffin oil. This method has proven very satisfactory, and luxuriant growths of active treponemata are secured uniformly within a 4 weeks' period of incubation.

After removal of the paraffin oil the fluid cultures in each tube were removed to sterile flasks with particular care not to break up the solid media and to remove the whitish sediment of treponemata which collects in the angle between the slant of egg media and the wall of the test-tube. After shaking the emulsions with sterile glass beads and centrifuging briefly, they were ready for use. Living cultures were employed throughout, and the emulsions were of such

density that each microscopic field examined by dark-field illumination showed from 12 to 30 treponemata.

Technique.—The macroscopic test was employed in exactly the same manner as in the previous study by Kolmer, except that lower dilutions were used; namely, 1:2, 1:5, 1:10, 1:20, 1:40, and 1:80.

1 cc. of treponema emulsion was used in each test, the serum being diluted in 1 cc. of sterile saline solution; the total volume, therefore, being 2 cc. with this amount of culture, a sufficient number of spirochetes were present to yield unmistakable agglutination in positive reactions. The first dilution (1:2) was prepared by adding 1 cc. of emulsion to 1 cc. of serum; further dilutions of serum in 1 cc., as 1:2.5, 1:5, 1:10, 1:20, and 1:40, were prepared, which after the addition of 1 cc. of emulsion were doubled in volume.

All the sera were unheated in dilutions varying from 1:2 to 1:80. After the preparations were set up the tubes were incubated for 2 hours at 37°C. and then placed in the refrigerator over night, final readings being made the next morning.

In every instance culture controls in normal saline solution were included; there were no evidences of spontaneous agglutination. As pointed out in the former communication, two or three spirochetes may be found in a loose clump, but in no instance were the large, clearly defined clump of spirochetes found, as in positive agglutinations.

In all tests a microscopic examination with the dark-field illuminator was made. Occasionally a light precipitate due to constituents in the culture medium may collect in the bottom of the small test-tubes, which macroscopically may be mistaken for agglutinated spirochetes; furthermore, the emulsions were not of sufficient density when diluted with an equal quantity of the serum dilution to render reliable a macroscopic reading of the reactions. For these reasons, we have examined each tube microscopically, removing the sediment or supernatant fluid or both with a fine capillary pipette and examining a drop by dark-field illumination.

In every instance the controls and negative reactions showed the presence of a large number of spirochetes, a few still motile, and all free from clumping. In a few instances two or three spirochetes were found in clumps, but there was no sedimentation and this was not

considered as an evidence of agglutination. In all positive reactions a drop of the sediment examined by dark-field illumination showed the presence of large clumps of spirochetes which were in most instances non-motile, although a few motile organisms were to be found at the edge of a clump, or entirely free. In the higher dilutions where agglutination was incomplete, free active spirochetes were found in the supernatant fluid and clumps in the sediment.

Wassermann Reactions.—All sera were submitted to the Wassermann reaction with three lipoidal antigens; namely, an alcoholic extract of human heart reenforced with cholesterol (C.H.), an alcoholic extract of syphilitic liver (S.), and an extract of acetone-insoluble lipoids from human heart muscle (A.).

With a portion of the sera an antigen of *Treponema* in the form of luetin was employed; this antigen was titrated before each experiment and used in an amount equivalent to one-third of the anti-complementary dose.

All reactions were read immediately after the secondary period of incubation.

RESULTS.

Normal Human Sera.—The results observed with twelve sera are shown in Table I.

Half of these sera showed no evidence of agglutination in the lowest dilution; namely, equal parts of undiluted serum and emulsion. As pointed out above, however, we have not designated as agglutination the loose clumps of two or three spirochetes which are occasionally encountered in tests with normal sera and in the controls.

In 50 per cent of these sera there were some evidences of agglutination in the lowest dilution, but none in dilutions of 1:5 and higher.

In our opinion agglutination of culture *Treponema pallidum* by human sera must occur in dilutions of 1:5 or higher before any significance may be attached to the result from the standpoint of syphilis.

Sera of Non-Syphilitic Individuals Suffering with Other Infections.—The results observed with eight of these sera are shown in Table II and are similar to those observed with the sera of normal persons shown in Table I. Several of these patients had moderate fever at the time the tests were made, but this factor and the presence of

TABLE I.

Agglutination of Treponema pallidum by the Sera of Normal Individuals.

Case No.	Wassermann reaction.			Agglutination.					
	C. H.*	S.†	A.‡	1:2	1:5	1:10	1:20	1:40	1:80
1	-§	-	-	-	-	-	-	-	-
2	-	-	-	±	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	-	-	+	-	-	-	-	-
5	-	-	-	±	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	+	-	-	-	-	-
10	-	-	-	±	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	-	-	±	-	-	-	-	-

* Cholesterolized alcoholic extract of human heart.

† Alcoholic extract of syphilitic liver.

‡ Acetone-insoluble lipoids of human heart.

§ - indicates absence of agglutination; resembles the salt solution controls.

± indicates slight but unmistakable agglutination characterized by numerous clumps of spirochetes; free spirochetes also occur in the supernatant fluid.

+ indicates well marked agglutination.

TABLE II.

Agglutination of Treponema pallidum by the Sera of Non-Syphilitic Individuals.

Case No.	Diagnosis.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Tuberculosis of penis.....	-	-	-	+	-	-	-	-	-
2	“ “ skin.....	-	-	-	-	-	-	-	-	-
3	“ “ lungs.....	-	-	-	-	-	-	-	-	-
4	Typhoid fever.....	-	-	-	±	-	-	-	-	-
5	Lobar pneumonia.....	-	-	-	±	-	-	-	-	-
6	Pyosalpingitis.....	-	-	-	-	-	-	-	-	-
7	Pregnancy.....	-	-	-	-	-	-	-	-	-
8	“	-	-	-	+	-	-	-	-	-

other antibodies in the sera, notably typhoid agglutinin, had no effect upon the spirochetes.

Primary Syphilis.—The results observed with five sera are shown in Table III.

TABLE III.

Agglutination of Treponema pallidum by the Sera of Patients in the Primary Stage of Syphilis.

Case No.	Treatment.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	1 dose arsenobenzol	++++*	++	++	≠	—	—	—	—	—
2	2 doses “	++++	++	+++	+	≠	—	—	—	—
3	1 dose “	++	—	—	+	≠	—	—	—	—
4	None	+	—	—	—	—	—	—	—	—
5	“	++	—	+	≠	—	—	—	—	—

* +++++ indicates complete inhibition of hemolysis (strongly positive); +++, 75 per cent inhibition of hemolysis (moderately positive); ++, 50 per cent inhibition of hemolysis (weakly positive); +, 25 per cent inhibition of hemolysis (very weakly positive); ≠, 10 per cent inhibition of hemolysis (doubtfully positive).

The first three patients were tested late in the primary stage and after treatment with arsenobenzol had been instituted; Cases 4 and 5 were tested about 2 weeks after the appearance of the chancre and before the treatment was begun.

In all cases the Wassermann reaction was positive; in Nos. 3 and 4 the reaction was positive with the cholesterolized extract only.

While we cannot draw conclusions from the examination of so few cases in this stage of syphilis, it would appear that traces of agglutinin for *Treponema pallidum* may be found in the serum in certain cases late in the primary stage. This opinion is based upon the higher percentage of partial agglutinations in the 1:2 dilution as compared with the sera of normal persons, and upon the occurrence of partial agglutination in the 1:5 dilution in two of the five sera.

Secondary Syphilis.—The results observed with the sera of twelve persons are shown in Table IV.

TABLE IV.
Agglutination of Treponema pallidum by the Sera of Patients in the Secondary Stage of Syphilis.

Case No.	Treatment.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	1 dose arsenobenzol	++++	++	++	+	+	±	-	-	-
2	1 " "	+	-	-	+	-	-	-	-	-
3	None	++++	++++	++++	+	±	-	-	-	-
4	2 doses arsenobenzol	++++	++++	++++	±	-	-	-	-	-
5	2 " "	++++	++	++++	-	-	-	-	-	-
6	2 " "	++++	+++	++++	+	+	+	+	-	-
7	Mixed (2 wks.)	++++	++++	++++	+	±	-	-	-	-
8	None	++++	++++	++++	+	-	-	-	-	-
9	6 doses arsenobenzol	-	-	-	-	-	-	-	-	-
10	4 " "	+++	++	++	+	±	-	-	-	-
11	None	++++	++++	++++	+	+	-	-	-	-
12	"	++++	++++	++++	+	+	±	-	-	-

All but four of these patients were tested after treatment had been instituted; all but one (Case 9) gave positive Wassermann reactions.

The sera of seven patients showed the presence of agglutinin in dilutions of 1:5 and higher; in no instance, however, was agglutination found in dilutions higher than 1:20.

These results appear to show quite definitely that agglutinins for *Treponema pallidum* are present in the sera of a proportion of cases of syphilis in the secondary stage.

Tertiary Syphilis.—The results observed with the sera of thirty-seven persons regarded as being in the tertiary or late latent stages of syphilis are shown in Tables V and VI.

In Table V are shown the results of agglutination tests with the sera of twenty-one persons regarded clinically as being well developed cases of paresis and of one person in whom the clinical diagnosis was doubtful paresis.

All but one of these sera agglutinated *Treponema pallidum*, and in the majority of instances in dilutions of 1:5 and higher. One serum caused well marked agglutination in dilutions as high as 1:80; the majority showed agglutination in dilutions varying from 1:5 to 1:40.

TABLE V.

Agglutination of Treponema pallidum by the Sera of Patients Suffering with Paresis.

Case No.	Diagnosis.	Wassermann reaction.				Agglutination.					
		Luetin.	C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Paresis.....	-	++++-	+	+++++	+	+	+	+	-	-
2	".....	±	+++++	++	+++++	+	-	-	-	-	-
3	".....	±	+++++	±	+++++	+	+	+	-	-	-
4	".....	±	+++++	+++++	+++++	+	+	-	-	-	-
5	".....	-	+++++	+++++	+++++	+	+	+	-	-	-
6	".....	-	+++++	+	++	+	+	+	+	+	-
7	".....	-	+++++	+++++	+++++	+	+	+	+	-	-
8	".....	+	+++	-	-	+	+	+	+	+	-
9	".....	-	+	+	+	+	+	+	+	+	-
10	".....	+	+++++	+++++	+++++	+	+	+	+	+	+
11	".....	+	+++++	+++++	+++++	+	+	+	-	-	-
12	".....	-	±	-	-	+	+	+	+	-	-
13	".....	-	+++++	+++++	+++++	+	+	+	-	-	-
14	".....	-	+++	+++	+++	+	+	+	-	-	-
15	".....	-	++	++	++	-	-	-	-	-	-
16	".....	+	+++++	+++++	+++++	+	±	-	-	-	-
17	".....	+	+++++	+++++	+++++	+	+	+	+	+	-
18	".....	-	+++++	+++++	+++++	+	+	±	-	-	-
19	".....	-	+++++	+++++	+++++	+	+	+	-	-	-
20	".....	-	+++++	+++++	+++++	+	+	+	-	-	-
21	".....	+	+++++	+++++	+++++	+	+	+	+	+	-
22	" (doubtful)...	-	-	-	-	+	+	±	-	-	-

In Table VI are shown the results observed with a group of cases yielding positive Wassermann or luetin reactions or both and regarded clinically as being syphilitic.

None of the five cases regarded clinically as dementia præcox of different types gave positive Wassermann reactions, although all these cases yielded a papular or pustular luetin reaction, and in three of them there was well marked agglutination of *Treponema pallidum*.

The results of these agglutination tests with the sera of patients in the late stages of syphilis show that agglutinin is especially likely to be found in the highest percentages of cases and in largest amounts at this period of the disease.

TABLE VI.
Agglutination of Treponema pallidum by the Sera of Patients in the Tertiary Stage of Syphilis.

Case No.	Diagnosis.	Luetin skin test.*	Wassermann reaction.			Agglutination.					
			Luetin.	C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40
1	Tabes dorsalis.....	++	-	++	-	-	+	+	+	-	-
2	Taboparesis.....	+	-	++++	++++	++++	+	+	+	-	-
3	".....	+	-	++++	++++	++++	+	+	+	-	-
4	Cerebrospinal syphilis....	++	++	++++	++++	++++	+	+	+	-	-
5	".....	=	-	++++	++++	++++	+	+	+	-	-
6	Dementia præcox.....	++	-	-	-	-	+	+	=	-	-
7	" ".....	+	-	-	-	-	+	+	+	-	-
8	" ".....	+	-	-	-	-	=	-	-	-	-
9	" ".....	++	-	-	-	-	+	+	+	+	+
10	" ".....	+	-	-	-	-	=	-	-	-	-
11	Depressive mania.....	+	-	+	-	-	+	+	+	+	-
12	" ".....	+	-	++	-	-	+	+	-	-	-
13	Aortic disease.....	0	+	++++	++++	++++	+	+	+	-	-
14	" aneurysm.....	0	-	++++	+	++	+	+	-	-	-
15	Gumma of brain.....	0	-	++++	++	+++	+	+	+	+	-

* ++ indicates pustular reaction; +, well defined papular reaction; =, slight papular reaction.

Agglutination and Complement Fixation.

An analysis of the results of agglutination and complement fixation tests with the sera of 54 persons yielding positive Wassermann or luetin reactions or both and regarded clinically as syphilitic, shows the following:

1. The Wassermann antibody seems to appear in the blood serum before demonstrable amounts of agglutinin.
2. The Wassermann antibody appears to reach a much higher degree of concentration in the blood serum.
3. The Wassermann antibody may be present while sufficient agglutinin to produce agglutination in dilutions of 1:5 and higher is absent, dilutions lower than 1:5 being unreliable because of the possibility of agglutination in a dilution of 1:2 by the sera of normal persons. Of the 54 sera from all stages of syphilis yielding positive

Wassermann reactions agglutination in dilutions of 1:5 and higher was absent in 14 sera or 26 per cent.

4. On the other hand, in 4 sera, or about 7 per cent of this series, demonstrable amounts of agglutinin were present and the Wassermann antibody was absent.

5. Of 37 patients regarded as being in the later stages of syphilis (Tables V and VI), 31 yielded positive Wassermann reactions and 8 positive and 3 doubtfully positive reactions with an antigen of luetin; all the sera yielding positive reactions with the antigen of luetin showed the presence of a demonstrable amount of agglutinin; on the other hand, a number of sera showing the presence of agglutinin did not absorb complement with the luetin antigen.

SUMMARY.

The results observed with this strain of *Treponema pallidum* may be stated as follows:

1. Normal human serum did not agglutinate this culture of *Treponema pallidum* in dilutions of 1:5 or higher; equal parts of treponema culture and normal serum (dilution 1:2) resulted in agglutination in about 50 per cent of the sera. With the strain of *Treponema pallidum* used in this study specific agglutination was not considered as having occurred unless observed in dilutions of 1:5 or higher.

2. The agglutinating power of the sera of non-syphilitic persons suffering with various infections was not higher than that of normal sera.

3. Traces of specific agglutinin for this culture of *Treponema pallidum* were found in the sera of persons late in the primary stage of syphilis.

4. In the secondary stage of syphilis about 58 per cent of sera showed the presence of a demonstrable amount of agglutinin in dilutions of 1:5 and higher; in no instance, however, was agglutination in evidence in dilutions higher than 1:20.

5. In tertiary and latent syphilis, mostly infections of the central nervous system, agglutination in dilutions of 1:5 and higher was found with about 84 per cent of sera. The highest concentration of *pallida* agglutinin in the blood serum was found in the later stages of syphilis.

6. The formation of agglutinin for culture *Treponema pallidum* in human syphilis is probably tardy and does not reach a state of high concentration.

7. There was no direct relation between the presence of the Wassermann antibody and agglutinin for this strain in the sera of syphilitics. The former appears in the body fluids earlier and is probably in higher concentration in all stages of syphilis; while agglutination is most likely to occur with Wassermann positive sera, it may be occasionally observed with sera yielding negative Wassermann reactions. Likewise strongly Wassermann positive sera may not contain demonstrable amounts of agglutinin.

8. It is probable that different strains of *pallida* vary in susceptibility to agglutination. For example, in the first investigation on agglutinins for *Treponema pallidum* by Kolmer, the sera of syphilitics in different stages of the disease did not cause agglutination of one of Noguchi's strains in dilutions of 1:20 and higher, whereas the strain used in the present study was frequently agglutinated in this dilution by sera from a similar group of patients. As shown by Zinsser, Hopkins, and McBurney, virulent *pallida* from human lesions resist agglutination to a remarkable degree.

9. While further studies are necessary to establish the practical value of agglutination in the diagnosis of human syphilis, we believe that with a suitable culture or cultures well marked agglutination of *Treponema pallidum* in dilutions of 1:5, or better 1:10 and higher, indicates *pallida* infection and may prove of value in the diagnosis of isolated cases, particularly in the later stages. Investigations bearing upon the relation of agglutinins for culture *pallida* to immunity in syphilis will be given in a separate communication (16).

BIBLIOGRAPHY.

1. Kolmer, J. A., Concerning Agglutinins for *Treponema pallidum*, *J. Exp. Med.*, 1913, xviii, 18-24.
2. Hoffmann and von Prowazek, cited by Neisser and Bruck in Beiträge zur Pathologie und Therapie der Syphilis, Berlin, 1911, 205; also *Arb. k. Gsndtsamte.*, 1911, xxxvii, 205.
3. Herxheimer, K., and Löser, Ueber den Bau der *Spirochaete pallida*, *Munch. med. Woch.*, 1905, liii, 2212-2213.

4. Hoffmann, E., Mitteilungen und Demonstrationen über experimentelle Syphilis, *Spirochaete pallid* und andere Spirochaetenarten, *Dermat. Z.*, 1906, xiii, 561-565.
5. Brönnum, A., and Ellermann, V., *Spirochaete pallida* in den inneren Organen bei Syphilis hereditaria, *Deutsch. med. Woch.*, 1905, xxxi, 1757-1758.
6. Babes, V., and Panea, J., Ueber pathologische Veränderungen und *Spirochaete pallida* bei congenitaler Syphilis, *Berl. klin. Woch.*, 1905, xlii, 865-869.
7. Metchnikoff, E., and Roux, E., Etudes expérimentales sur la syphilis, *Ann. Inst. Pasteur*, 1905, xix, 673-698.
8. Landsteiner, K., and Mucha, V., Beobachtungen über *Spirochaete pallida*, *Centr. Bakteriol., 1te Abt., Ref.*, 1907, xxxix, 540-541.
9. Landsteiner and Mucha, Zur Technik der Spirochaetenuntersuchung, *Wien. klin. Woch.*, 1906, xix, 1349-1350.
10. Zabolotny, D., and Maslakowetz, Beobachtungen über Beweglichkeit und Agglutination der *Spirochaete pallida*, *Centr. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 532-534.
11. Uhlenhuth, P., and Mulzer, P., Beiträge zur experimentellen Pathologie und Therapie der Syphilis mit besonderer Berücksichtigung der Impf-Syphilis der Kaninchen, *Arb. k. Gsndhsamte.*, 1913, xliv, 307-530.
12. Nakano, H., Über Immunisierungsversuche mit Spirochaeten-Reinkulturen, *Arch. Dermat. u. Syph.*, 1913, cxvi, 265-280.
13. Kissmeyer, A., Agglutination der *Spirochaete pallida*, *Deutsch. med. Woch.*, 1915, xli, 306-308.
14. Zinsser, H., and Hopkins, J. G., Antibody Formation against *Treponema pallidum*—Agglutination, *J. Exp. Med.*, 1915, xxi, 576-583.
15. Zinsser, H., Hopkins, J. G., and McBurney, M., Studies on *Treponema pallidum* and Syphilis. IV. The Difference in Behavior in Immune Serum between Cultivated Non-Virulent *Treponema pallidum* and Virulent Treponemata from Lesions, *J. Exp. Med.*, 1916, xxiii, 341-352.
16. Kolmer, J. A., and Broadwell, S., Jr., The Relation of the Luetin Skin Reaction to Immunity in Syphilis. The Treponemicidal Action of the Serum in Human Syphilis, *J. Immunol.* (in press).