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COMPLEMENT FIXATION, PRECIPITIN, ADHESION, MERCURIC CHLORIDE AND WASSERMANN TESTS IN EQUINE TRYPANOSOMIASIS OF PANAMA (MURRINA)[1]

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In the Panamanian region there are sporadic outbreaks of trypanosomiasis in horses and mules. During the spring of 1931 we were afforded the opportunity of studying the serology of this infection in an untreated herd of mules and in a herd of horses which had undergone a series of treatments with Bayer 205 and tryparsamide. This serological investigation was carried out with two objects in view. It allowed us, in the first place, to compare the results of complement fixation, precipitin, red blood cell adhesion, Wassermann and mercuric chloride tests, and in the second place, to evaluate these various serological tests in terms of the blood findings with a view toward perfecting a diagnostic test.

LITERATURE REVIEW

Darling (1910) made the first scientific record of equine trypanosomiasis in Panama. He adopted one of the local names, murrina, for the disease and named the causative organism *Trypanosoma hippicum.* More recently, Clark, Casserly and Gladish (1933) have investigated the disease extensively. It is unnecessary to describe the clinical picture of the disease because a positive diagnosis rests solely upon the demonstration of the parasites in the peripheral blood.

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A systematic review of the results of various serological tests in different trypanosomiases has been given by the senior author (1929) and need not be repeated here. It will suffice to point out that complement fixation has assumed an outstanding usefulness in the case of dourine and that other serological tests, in particular, agglutinin and precipitin tests, appear promising from preliminary work and may well be perfected. As far as we are aware, none of these tests have been applied specifically to the Panamanian trypanosomiasis of horses and mules.

Since the publication of the review referred to in the preceding paragraph, two interesting tests have been developed, a specific serological test and a non-specific flocculation test.

The specific serological test grew out of Rieckenberg's (1917) blood platelet adhesion test. In 1928 Leupold noted an adhesion between the trypanosomes and red blood cells in some of her blood platelet tests. In 1930 Duke presented an account of this test in preliminary form and in the same year Duke and Wallace (1930) investigated the phenomenon both from the standpoint of its value in diagnosis and its mechanism. Working with *T. rhodesiense, T. gambiense* and several allied strains, they concluded that the adhesion phenomenon occurs and may persist for more than two years in infected animals, but is so irregular and uncertain in its appearance that a single or even a number of negative observations do not exclude trypanosomiasis, while a positive reaction indicates recent or actual infection with a trypanosome of the same group (not necessarily of the same species); that the test may be positive after the immediate treatment with drugs, but becomes negative in the course of a few months and that the mechanism is in some way associated with the red cells of primates. The test as used by these investigators involves the addition of one drop of a trypanosome suspension to one drop of equal parts of blood and 2 per cent sodium citrate. If the blood comes from an infected animal, red blood cells (also, occasionally, blood platelets) adhere to the trypanosomes within ten or fifteen minutes. In 1931, Wallace and Wormall concluded that, in addition to the antibody-like constituent, the red cells of *primates* and a complement-like component were necessary in the adhesion test when one is testing for *T. rhodesiense* and *T. gambiense.*

The non-specific test is the mercuric chloride flocculation test of Bennett and Kenny (1928). These investigators, in working with trypanosomiasis of camels (*T. soudanense*), found that when one drop of serum was added to 1 cc of a 1:20,000 dilution of mercuric chloride a pronounced opacity occurred if the serum originated from infected animals,

whereas no change took place if it came from normal animals. Ordinarily, readings were made fifteen minutes after the addition of the serum to the mercuric chloride solution, but readings of an hour or more were approximately identical. Their preliminary results indicated that the majority of infected animals could be detected a fortnight after infection. In any case, the test seemed quite superior to the formol gel reaction. Bennett (1929) and Horgan and Bennett (1929) confirmed the delicacy of the mercuric chloride test and the latter investigators concluded that the mechanism of the positive reaction was referable to a relative and absolute increase of the serum-euglobulin. Wilson (1930), working with *T. rhodesiense* in man, concluded that the test was not specific but was valuable when strongly positive (5+ in 1:20,000 dilution in fifteen to twenty minutes) or when moderately positive if the patient exhibited clinical signs and symptoms of sleeping sickness or came from a sleeping sickness area. After several treatments with Bayer 205 or tryparsamide the test became negative.

MATERIAL AND METHODS

Complement fixation tests were not carried out by us. Samples of blood which in most cases were taken at the same time as the ones used in this paper were carbolized to 0.5 per cent and forwarded in refrigeration to the Bureau of Animal Industry and the Army Medical Center of Washington, D. C. These specimens were then tested by Drs. J. R. Mohler or J. S. Buckley (series B and D) and Major R. A. Kelser (series A and C) respectively. Both laboratories used antigens prepared from *T. equiperdum* and *T. hippicum* and their results were quite similar although a few discrepancies occurred. Through the kindness of Dr. H. C. Clark, for whom these tests were run, we have been able to use their findings (see Clark, Casserly and Gladish (1933).

Precipitin tests were carried out with aqueous antigens prepared by the method of Watson (1920) from (1) a strain of *T. hippicum* originally isolated by Dr. Clark from an animal afflicted with the Panamanian disease and (2) a strain of *T. equiperdum* furnished by the Department of Agriculture. In accordance with this procedure, trypanosomes from rats, sacrificed at the height of their infections, were separated as far as possible from red blood cells and serum by successive centrifugations, were killed and preserved in twice their volume of a preserving fluid consisting of 90 parts 0.85 per cent NaCl, 10 parts pure neutral glycerine and one-tenth part formalin. The largest batches of antigens were prepared in Chicago and were shipped to Panama, but a few were prepared

in Panama from an infected *rhesus* monkey or guinea pigs. The *T. equiperdum* antigens were as a rule obtained from rats with heavier infections and hence, as will be seen later, were stronger and more efficient. All, however, gave essentially similar results and retained their antigenic power for at least three months. Alcoholic antigens were also used, but were not found to be as satisfactory as the aqueous ones. They were prepared by adding to the isolated trypanosomes, obtained as described above, an equal quantity of absolute alcohol.

The ring test with small tubes of 4 mm. bore was used. Approximately 0.1 cc. of serum was carefully overlaid with an equal amount of antigen or control solution, and after an hour at room temperature the tubes were examined for a ring of precipitate at the junction of the two liquids. In general, each test consisted of 3 tubes in each of which was pipetted the same amount of the same serum and in the first and second of which was overlaid the undiluted and 1:5 antigen, respectively, and in the third of which was overlaid the diluent control, i.e., the solution, which was mixed with the trypanosomes to make the antigen.

In the blood adhesion test, the technic of Duke and Wallace was essentially used. When blood was taken from the horses or mules, approximately 1 cc. was placed before clotting in a vial containing 1 cc. of 2 per cent sodium citrate. This mixture was brought to the laboratory and tested within a few hours. The test was carried out by mixing a drop of citrated blood with a similar citrated blood mixture from a guinea pig infected with *T. hippicum*, on a microscopic slide, and after ten minutes observing 100 trypanosomes to ascertain whether any of them showed blood cells adhering to them. Ten per cent or more of trypanosomes with adhering red blood cells constituted a positive test. In some cases a trypanosome may have had one red cell adhered to it, in other cases it may have been covered with red cells giving the appearance which Duke and Wallace termed *caddis* forms. All of these, without distinction, were considered adhesion forms. We found, as did Duke and Wallace, that the final mixture should contain only a few trypanosomes per microscopic field. If too many trypanosomes were present, the individual organisms were difficult to study and if too few were present, the tests consumed too much time.

Mercuric chloride tests were carried out according to the method of Bennett and his co-workers. For comparative purposes we used four Wassermann tubes, the first three containing mercuric chloride in dilutions of 1:15,000, 1:20,000 and 1:25,000, respectively, and the fourth containing distilled water. In general we found that the only significant

results were obtained in the second and third tubes. The tube containing the 1:15,000 dilution tended to flocculate with many serums and the tube containing distilled water flocculated only with those serums which were strongly reactive.

Wassermann tests with the Noguchi modification were performed at the laboratory of the Board of Health in the Canal Zone under the direction of Dr. L. B. Bates. Two units each of amboceptor and complement were used in each tube. To test each serum, two antigens, one-half saturated with cholesterin, were used.

THE UNTREATED HERD

The untreated herd consisted of 101 mules and 2 horses owned by the Standard Fruit and Steamship Company and located on their Escobal Farm on the west bank of Gatun Lake in the Canal Zone. (An extra infected untreated mule from another source is included in some of the tests.) The infection was not present in August, 1930, according to thick blood film surveys, but had made its appearance by April, 1931. On April 20, 1931, 38 blood samples were collected for precipitin, adhesion and mercuric chloride tests, and thick blood films for examination were made from the whole herd. On May 4, 1931, 102 blood samples were collected for precipitin, complement fixation, adhesion, mercuric chloride and Wassermann tests and for thick blood film examinations. Additional blood film examinations for trypanosomes were made from all the horses on May 4, 11 and 13, 1931. The trypanosomes were very scarce. In fact, in 8 of the 16 infected animals, trypanosomes were only encountered on one day and then in very small numbers. In the balance of the infected animals they were never found in more than three examinations.

Complement fixation tests on the untreated herd

The results of the complement fixation tests are considered first because this test has been by far the most widely used in the diagnosis of other equine trypanosomiases and is very generally relied upon in the diagnosis of dourine.

In the untreated herd at Escobal four series of tests were carried out on the serums collected May 4, 1931, with *T. equiperdum* and *T. hippicum* antigens. The different series are so similar that

they do not need to be considered in detail (table 1). Series A which contained one positive serum not included in the other series was performed with a *T. hippicum* antigen. Aside from 3 serums from negative animals, which were anticomplementary, serums from 15 known infected animals gave 14 positive (13++++ and 1+++) and one negative reaction and serums from 84 animals negative in thick film gave 79 negative and 5 positive reactions (2++++, 1+++ and 2+). It is, of course, doubtful whether the two single plus reactions should really be considered positive. In comparing the various series, it is interesting to note that the serum from the one known infected horse giving a negative test was the same one which was negative in all the other series. Furthermore, of the serums from the 5 mules showing no trypanosomes

TABLE 1

Correlation between thick film examinations for T. hippicum and 4 series of complement fixation tests on the same serums collected May, 1931, from the untreated herd

TRYPANOSOMES IN THICK FILM	COMPLEMENT FIXATION TESTS															
	T. hippicum antigen								*T. equiperdum* antigen							
	A: 99 serums				B: 100 serums				C: 98 serums				D: 100 serums			
	4+	3+	1+	0	4+	2+	1+	0	4+	2+	1+	0	4+	2+	1+	0
+	13	1		1	12	1		1	13			1	13			1
−	2	1	2	79	1	2	2	81	2	1	1	80	1	1	3	81

in their blood, which gave various degrees of positiveness for complement fixation, the following is true: of the 2 giving 4+ reactions one was uniformly 4+ in all series and the other was 4 + in series C but negative in series B and D; the one giving a 3+ reaction was 2+ in series C and 1+ in series B and D; and of the two giving a + reaction, one was uniformly negative in the other three series and one was 1+ in one other series and 2+ in two. In addition, one serum from a mule negative in thick film was 2+ in series B, was 1+ in series D, and − in series A and C.

Precipitin tests on the untreated herd

The most promising specific serological test, aside from the complement fixation test, was the precipitin reaction with aqueous

antigens. The alcoholic antigens, as used in the ring test, gave so few strongly positive reactions that they need not be considered further. This lack of reactivity is probably connected with the dilution-factor rather than the lack of an alcohol-soluble specific principle inasmuch as the test antigen had to be diluted 1:10 with saline in order that no coagulation of the serum would occur at the junction of the two liquids.

The results of tests on the serums from 102 animals are given in table 2. With the *equiperdum* antigen, 15 serums from known infected animals gave 15 positive (9++++, 3+++ and 3++) reactions and 87 serums from animals in whose blood trypanosomes were not found gave 20 positive (1++++, 6++ and 13+) and 67 negative reactions. With the *hippicum* antigen, on the

TABLE 2

Correlation between thick film examinations for T. hippicum and 2 series of precipitin (ring) tests on the same serums collected May, 1931, from the untreated herd

TRYPANOSOMES IN THICK FILM	PRECIPITIN TESTS									
	T. hippicum aqueous antigen					*T. equiperdum* aqueous antigen				
	102 serums					102 serums				
	4+	3+	2+	1+	0	4+	3+	2+	1+	0
+	4	4	3	4		9	3	3		
−			1	15	71	1		6	13	67

other hand, there were neither as many strongly positive reactions nor quite as good a correspondence with infection and non-infection (table 2). In both series, the 1+ reaction, which shows a very weakly discernible ring, should probably be considered negative. The superiority of the *T. equiperdum* antigen is probably an expression of antigen-strength since, as stated in Materials and Methods, there were more trypanosomes in the *equiperdum* than in the *hippicum* antigen. Then, too, the group reaction between the two trypanosomes is very frequent.

Tests on some of the animals fourteen days previously, gave similar results. Thus, serums from 9 positive animals gave 2++++, 3+++, 2++ and 2+ reactions with the *T. hippicum* antigen and 2++++, 3+++ and 4++ reactions with the

T. equiperdum antigen while serums from 25 animals in whose blood trypanosomes were not found elicited 6+ and 19− reactions with the *T. hippicum* antigen and 1++, 6+ and 18− reactions with the *T. equiperdum* antigen. Twelve of the negative serums, however, when tested with the *equiperdum* antigen were cloudy and difficult to read.

Adhesion tests on the untreated herd

Two series of adhesion tests were performed on the Escobal herd, the first on 37 animals and the second on the whole herd and one additional infected mule from another herd. Although there are a few discrepancies, the tests correspond fairly well. In the

TABLE 3

Correlation between thick film examinations for T. hippicum and adhesion tests on 2 batches of serums collected March and May, 1931, respectively, from the untreated herd

TRYPANOSOMES IN THICK FILM	ADHESION TESTS SHOWING PER CENT OF ADHESION							
	37 serums				104 serums			
	100–71	70–41	40–10	9–0	100–71	70–41	40–10	9–0
+	2	5	1	2	3	4	9	
−		1	1	25		1	10	77

smaller series, serums from 10 known infected animals gave 8 positive, i.e., 10 per cent or more adhesion forms, and 2 negative reactions and from 27 animals in which trypanosomes had not been found gave 2 positive and 25 negative reactions (table 3). In the second and larger series serums from all of the 16 known infected animals gave positive tests, whereas serums from 88 animals with negative thick blood films gave 11 positive and 77 negative reactions. Interestingly enough the serum from one animal (no. 10), whose thick blood film was negative, was positive not only in all 4 series of the complement fixation and in one of the precipitin tests, but also in both adhesion tests, giving 60 per cent adhesion in one series and 40 per cent in the other. The 2 known infected animals which gave negative tests in the first series (3 and 8 per cent) gave positive tests in the second series (28 and 16 per cent).

Mercuric chloride tests on the untreated herd

The simplicity of performing this test intensified our interest in it. Accordingly, a great deal of work was done in trying to standardize it. Readings of the 4 tubes containing 1:15,000, 1:20,000 and 1:25,000 dilutions of mercuric chloride and distilled water, respectively, were made immediately, and one and 3 hours (and in a few cases eighteen hours) after the drop of serum had been added to each of the tubes. Furthermore, tests on 76 serums were carried out immediately after collection and at the expiration of one and six days; on 41 serums with fresh and old distilled water; on 5 serums with fresh and old solutions of mercuric chloride; on 4 serums containing in each series many, few or no red blood cells; and on 6 serums using varying sized drops of serum. The conclusions reached when the results of these various tests were analyzed are as follows: Positive serums collected within twenty-four to thirty-six hours are reactive, but gradually tend to become less so. The immediate and three-hour readings appear to be better than the one hour reading. This is probably due to the fact that the criteria in reading the immediate and the 3-hour reading depend on different aspects of the change taking place in the tubes of which the one-hour reading is intermediate, and therefore, difficult to evaluate. Since the immediate reading is the simplest to use in practice, we selected it in reporting the following data except where specifically denoted otherwise. It should be pointed out, however, that at the end of three hours the precipitate is fully formed and settled and its volume easier to determine. Freshly distilled water appears to be somewhat superior to old distilled water. A 1:100 dilution of mercuric chloride does not seem to deteriorate upon standing one month. It is better to make up the 1:15,000, 1:20,000 and 1:25,000 dilutions for the actual tests in about 150 cc. of distilled water to rule out irregularities as far as possible. Any red blood cells in the serum cause markedly more precipitation. A free running drop from a pipet with an aperture at its tip measuring approximately 0.5 mm. is sufficient to cause the precipitate to form, and, on the other hand, excessive amounts tend to inhibit the formation of the precipitate.

The procedure for reading the tests was somewhat as follows: Upon the addition of the drop of serum, each tube was shaken and the amount of cloudiness and precipitate noted. All tubes were then left undisturbed for one hour and again read; after the reading they were shaken. At the end of three hours, they were read without shaking and then ordinarily discarded.

The criteria for determining positiveness depended upon a precipitate in the tube, but the amount depended upon the time of the reading. Thus, a strongly positive reaction at the immediate reading showed a slight flaky precipitate causing cloudiness whereas at the three-hour reading it showed a decided precipitate which, after settling, occupied half the volume of the liquid. A weakly positive reaction (+) at the immediate reading showed a

TABLE 4

Correlation between thick film examinations for T. hippicum and the mercuric chloride test on 2 batches of serums collected March and May, 1931, respectively, from the untreated herd

TRYPANOSOMES IN THICK FILM	MERCURIC CHLORIDE TESTS					
	37 serums			104 serums		
	2+	1+	−	2+	1+	−
+	5	4	1		14	2
−	1	1	25	1	22	65

distinct precipitate causing cloudiness of the liquid, at the one-hour reading it showed a noticeable cloudiness of the liquid with some slight settling out of the precipitate, whereas at the three-hour reading it showed a precipitate which had settled out and at least covered the bottom of the tube. Intermediate grades were also encountered. The difference in amount of precipitate is clearly brought out by the following readings. In a test on April 21 of 10 positive serums collected April 20, the immediate reading gave 5++, 4+ and 1− readings, whereas the three-hour reading gave 9+++ and 1− readings. A negative test, on the other hand, might show a slight cloudiness at the immediate reading which became more intense as time went on, but it never showed an appreciable amount of precipitate and only a little,

if any, settled out by the end of 3 hours. Distinctly negative reactions with no change whatever upon the addition of the serum constantly occurred. Intermediate grades showed a slight opalescence.

Tests were made on two batches of serums not more than thirty-six hours old, of 37 and 104 serums, respectively. In the first series, of 10 serums from positively infected mules, 9 (5++ and 4+) were positive and 1 negative and of 27 serums from mules not found infected 2 (1++ and 1+) were positive and 25 were negative. In the second series, of 16 serums from positively infected mules, 14 (+) were positive and 2 were negative and of 88 serums from animals not found infected, 23 (1++ and 22+) were positive and 65 were negative. The first set of tests shows a very good correspondence between positiveness and infection, but the second is rather poor (table 4).

Wassermann tests on the untreated herd

Of the 103 Wassermann tests carried out on the serums collected in May from all of the members of this herd whether infected or uninfected, 102 were negative and only one was positive (++). Other tests on the one positive serum, which came from an animal found infected with many trypanosomes on April 20 and a smaller number on May 4, were as follows: Complement fixation 4+ in all series; precipitin 1+ and 2+ with *T. hippicum* and *T. equiperdum* antigens, respectively; adhesion + (16 per cent) and mercuric chloride +.

Infections other than trypanosomiasis in the untreated herd

In this herd, there was 1 animal in whose blood both trypanosomes and piroplasms were found and 6 animals uninfected with trypanosomes but in 5 of which piroplasms were found and in one of which microfilaria were found. These infections did not interfere with the specific tests nor did there seem to be more positive reactions with the non-specific mercuric chloride test than were recorded ordinarily, as may be seen by an examination of table 5. Thus, the multiple tests performed on the 2 bleedings of the animal which was infected with both trypanosomes and piroplasms were

strongly positive in all three of the specific tests and gave 2++ and 2+ reactions in the mercuric chloride test, whereas the serums of the animals found infected with either piroplasms or filaria, but uninfected with trypanosomes, were all negative in the complement fixation and adhesion tests, showed 1 weakly positive and 15 negative precipitin reactions and 5 weakly positive and 12 negative mercuric chloride tests. The data are consistent in showing that these extraneous infections, provided there be no trypanosome-infection present, do not cause the serums to react positively, and therefore, do not interfere with the tests in their applicability to detect trypanosomiasis.

TABLE 5

Results of multiple serological tests for trypanosomiasis on 2 batches of serums collected March and May, 1931, from seven animals of the untreated herd in whose blood either piroplasms or filaria were found

THICK FILM		TESTS										
		Complement fixation		Precipitin				Adhesion		Mercuric chloride		
		28 tests on 7 serums		20 tests on 7 serums				10 tests on 7 serums		21 tests on 7 serums		
Trypanosomes	Piroplasm or filaria	4+	−	4+	3+	+	−	+	−	++	+	−
+	+	4		1	3			2 (60%)		2	2	
−	+		24			1	15		8		5	12

Correlation of all of the serological tests on the untreated herd

Since complement fixation, precipitin, adhesion and mercuric chloride tests were carried out on 103 serums obtained at the same time in May, 1931, they have been correlated in table 6. In tabulating these data, 4, 3 and 2 plus reactions were considered positive and 1 plus and − were considered negative in the complement fixation and precipitin tests, and all grades of 1 plus or above and of 10 per cent or above were considered positive in the mercuric chloride and adhesion tests, respectively. The most interesting facts about this table are: that 12 of the 15 serums from animals in which trypanosomes were found were positive in all four tests and that the other 3 were positive

in three of the tests (2 were negative with the mercuric chloride and 1 with the complement fixation); and that of the 88 serums from animals negative in thick film 1 was positive in all four tests, 4 were positive in two tests, 30 were positive in one test and 53 were negative in all four tests. Although complement fixation tests were not carried out on the serums collected in March, the data on the three other tests are completely in accord with the data just given in that the serums from known infected animals were more prone to be positive in all of the tests and the serums from animals supposedly free of trypanosomes were prone to be negative in all of the tests.

TABLE 6

Correlation between the thick film examinations for T. hippicum and all of the tests performed on serums collected May, 1931, from the untreated herd

	TESTS—103 SERUMS										
Complement fixation....	+	+	−	−	−	+	+	−	−	−	−
Precipitin.....	+	+	+	+	−	−	−	+	−	−	−
Adhesion.....	+	+	+	+	+	−	−	−	+	−	−
Mercuric chloride.....	+	−	+	−	+	+	−	−	−	+	−
TRYPANOSOMES IN THICK FILM											
+	12	2	1								
−	1			1	2	1	1	4	7	18	53

THE TREATED HERD

The treated herd consisted of 105 horses and 17 mules or burros of the stock breeding farm of the Panama Canal at Miraflores situated on the west side of Miraflores Lake in the Canal Zone. The infection was first discovered by Dr. H. C. Clark in December, 1929, and has been closely followed by him since then. Monthly examinations by the thick film method were made of all the herd, and occasionally, more frequent examinations were made on certain animals showing clinical symptoms. Thus, in March 1931, 101 of the animals had been examined from 18 to 21 times, and the balance from 5 to 16. The small number of examinations denoted the arrival of newly born animals in the herd. Furthermore, on January 27, 1931, a guinea pig was inoculated with blood

from each animal which had previously been found infected with the result that only one animal (No. 88) was found still infected. Aside from this animal, none had been found positive since October, 1930, and the great majority had not been found positive since April. In fact, 19 of the 35 infected animals responded so immediately to treatment that trypanosomes were not found in their blood after January, 1930.

The herd was so valuable that the following treatments were given by Dr. T. L. Casserly: All members of the herd were treated intravenously with Bayer 205 on January 9, 1930, in doses of 4 grams for mature animals and 2 grams for colts and small animals; with tryparsamide, on January 16, 1930, in 5-gram doses; and with Bayer 205, on January 23, 1930, in doses averaging 3 grams for mature animals and 1.5 grams for colts and small animals. For the next two months individual animals were given additional doses if they showed clinical symptoms or positive blood films. The entire herd was again treated in April, 1930, and thereafter a few animals were given subsequent treatments.

A brief summary of the blood examinations discloses that of the 35 animals found infected, 19 showed trypanosomes in January, 1930, 11 in April, 1930, 4 in October, 1930, and 1 in January, 1931, whereas 87 never showed trypanosomes in their blood. In 8 of those which showed trypanosomes in their blood later than January, 1930, trypanosomes had been found previously, but in the balance they were discovered for the first time.

On May 11, 1931, the whole herd of 122 animals was bled for precipitin, adhesion, mercuric chloride tests and for blood examinations and on May 25, 41 were bled again for a second mercuric chloride test. On January 27, 1931, the herd was bled for two independent complement fixation tests and on March 11, 21 of the herd were re-bled and retested. Thus, the serums for the complement fixation tests were not collected from this herd at the same time as were those for the other tests, but they are fairly comparable since the animals had been treated for such a long time and since the majority of animals had not had trypanosomes in in their blood for from five to ten months.

Complement fixation tests on the treated herd

The two series of independent complement fixation tests on serums collected in January, 1931, showed no correspondence with previous infection in this treated herd (table 7). Thus, serums from 34 animals previously infected gave 1 (4+) positive and 33 negative reactions. These results might be explained on the basis that the animals had been cured by previous treatment. The disturbing occurrence seemed to be that serum from the one animal (no. 88) still showing trypanosomes in its blood was negative in both tests. Therefore, 22 of the animals whose serums showed certain discordances in the two series of tests or were of interest for other reasons were retested in two series in March. The results of these retests showed that 12 of the serums were

TABLE 7

Correlation between thick film examinations for T. hippicum and 2 series of complement fixation tests on the same serums collected January, 1931, from the treated herd

TRYPANOSOMES PREVIOUSLY IN THICK FILM	COMPLEMENT FIXATION TESTS—*T. hippicum* ANTIGEN						
	A: 121 serums				B: 121 serums		
	4+	+	±	0	4+	±	0
+	1		4	29	1	3	30
−		5	2	80	1	3	83

consistent with the previous tests. The other 10 were remarkably variable. Thus, from previously positive animals, serum 46 gave ±, −, 4+ and − in the 4 series, serums 30 and 88 were essentially negative in the first two series and 4+ in the last two series and among those animals never found infected serum 139 was 1+ in the first series and 4+ in the other three, serum 156 was 1+, −, 4+ and − in the four series and both serums 162 and 163 were 1+, ±, 4+ and 4+ in the four series, respectively. In the face of these results it is interesting to note that at least some of the serums when collected at the same time tended to give the same results in the independent tests—thereby showing that the variable behavior was inherent in the serums themselves and not a fault of the technic. Thus, aside from the serums consistent throughout,

serums 30, from among the previously positive animals, and 88, from the one animal still infected in January, were essentially negative by both tests when collected in January and 4+ by both tests when collected in March and serums 162 and 163 from supposedly uninfected animals were + and ± when collected in January and 4+ when collected in March.

Precipitin tests on the treated herd

Precipitin tests were carried out with an aqueous antigen of *T. hippicum* on 80 animals, 34 of which had been previously infected. The results, which may be found in table 8, show just about the same lack of reactivity as was recorded in the complement fixation tests of January. There were no strong reactions

TABLE 8

Correlation between thick film examinations for T. hippicum and the precipitin tests on serums collected May, 1931, from the treated herd

TRYPANOSOMES PREVIOUSLY IN THICK FILM	PRECIPITIN TESTS—T. HIPPICUM AQUEOUS ANTIGEN—80 SERUMS		
	2+	+	0
+	1	1	32
−	1	2	43

and the majority were frankly negative. Parenthetically, it may be noted that the test on No. 88, which was found infected in January, was negative.

Adhesion tests on the treated herd

In the adhesion test serums from 122 animals of this herd were all negative with the exception of two serums from previously infected horses and one from a supposedly uninfected animal. These showed 16, 17, and 59 per cent of adhesion forms, respectively. The test of No. 88 was negative.

Mercuric chloride tests on the treated herd

There were so many positive—and even strongly positive—mercuric chloride tests with serums from 122 animals of this herd that the same serums were retested and two weeks later 41 fresh

serums were obtained and tested twice. As may be seen by an examination of two of these series in table 9, the serums appeared quite liable to precipitate with the mercuric chloride. Thus, in the first series, of the 35 serums from animals previously infected, 16 were positive (4++ and 12+) and 19 negative and of the 87 from uninfected animals, 58 were positive (27++ and 31+) and 29 were negative. Similarly, in the second series, of the 9 serums

TABLE 9

Correlation between thick film examinations for T. hippicum and the mercuric chloride tests on 2 batches of serums collected May 11 and 25, 1931, from the treated herd

TRYPANOSOMES PREVIOUSLY IN THICK FILM	MERCURIC CHLORIDE TESTS					
	122 serums			41 serums		
	++	+	−	++	+	−
+	4	12	19	1	1	7
−	27	31	29	10	10	12

TABLE 10

Correlation between the thick film examination for T. hippicum and all of the tests performed on serums collected in January and May, 1931, from the treated herd

	TESTS—77 SERUMS					
Complement fixation	−	−	+	−	−	−
Precipitin	−	+	−	−	−	−
Adhesion	+	−	−	+	−	−
Mercuric chloride	+	+	−	−	+	−
TRYPANOSOMES PREVIOUSLY IN THICK FILM						
+		2	1	1	13	16
−	1	3			18	22

from animals previously infected, 2 were positive (1++ and 1+) and 7 negative and of the 32 serums from probably uninfected animals, 20 were positive (10++ and 10+) and 12 were negative.

Correlation of all of the tests on the treated herd

In striking contrast to the correlation between infection and positiveness for all of the four tests of interest in this paper in the

untreated herd, is the lack of such correlation in the treated herd. Thus, none of the serums whether from formerly positive or supposedly negative animals was positive in all or even in three of the tests and only 6 of the serums were positive for two tests, (table 10). Furthermore, as a rule, the complement fixation, precipitin and adhesion tests tended to be negative, whereas the mercuric chloride test tended to be positive. Thus, as may be seen in table 10, 37 of the 39 serums which were positive in some test were positive in the mercuric chloride test, whereas only 1, 5 and 2 serums were positive in the complement fixation, precipitin and adhesion tests, respectively. Moreover, even though the mercuric chloride test showed more positive reactions, it did not seem to correspond with previous infection, since of the 33 serums from formerly infected animals, 15 or 45 per cent were positive, whereas of the 44 from animals never found infected, 22 or 50 per cent were positive.

DISCUSSION

The most outstanding difference in the results of the two foregoing series of tests is the excellent correlation between infection and the serological tests in the untreated herd and the poor correspondence in the treated herd. The fact that the untreated herd consisted largely of mules and the treated herd of horses does not seem to be of importance because the mules in the treated herd showed the same inconsistent results as the horses. The lack of correlation between past infection and serological tests in the treated herd is all the more striking because it applies to all of the tests. A comprehensive explanation of how treatment could cause such discrepancies is not evident, but mass treatment such as was given would obviously cause some of the lack of correspondence. Thus, an infected animal might be cured, and therefore, give a negative serological reaction, or treatment of an animal lightly infected might hold down the infection so that it would never be detected but might not prevent a definite serological response.

In carrying out the adhesion tests, Duke and Wallace (1930) and Wallace and Wormall (1931) considered the red cells of pri-

mates as a necessary constituent of the set-up. We used, however, equine red cells and obtained results comparable with the other tests we studied and also with the blood findings. The use of the equine cells may account for the fact that only occasionally did we encounter such an extreme adherence of blood cells to the trypanosomes as Duke and Wallace and Wallace and Wormall described. On the other hand, the fact that we obtained such a comparable correlation indicates that equine cells are satisfactory in studying *T. hippicum*.

SUMMARY

The data presented give a comparison of complement fixation, precipitin, red blood cell adhesion and mercuric chloride tests with thick blood film findings in two herds of horses and mules some of each of which were infected with *T. hippicum*. One herd contained 103 animals which were never treated (tables 1 to 6), and the other contained 122 animals which were treated (tables 7 to 10).

The untreated herd showed a striking correlation between infection of the animal and positiveness of all the tests. Thus, in one series of 103 serums where parallel tests were run, of 15 serums from demonstrably infected animals 12 were positive in all four tests, and the other three were positive in three tests (2 were negative with the mercuric chloride and one with the complement fixation) and of 88 serums from animals in which trypanosomes were never found, one was positive in all four tests, four were positive in two tests (1 in precipitin and adhesion, 2 in adhesion and mercuric chloride and 1 in mercuric chloride and complement fixation), 30 were positive in one test (1, 4, 7 and 18 in complement fixation, precipitin, adhesion and mercuric chloride, respectively) and 53 were negative in all four tests (table 6). A few cases of piroplasm and one case of filaria did not cause pseudo-positive reactions in any of the tests (table 5). Another series of tests on approximately one-third of the herd furnished corroborative data. Wassermann tests on this herd gave 1 (++) positive and 102 negative reactions. In general, all of the specific tests (complement fixation, precipitin and adhesion) on the untreated herd were

more consistent and reliable (tables 1 to 3) than the non-specific mercuric chloride test (table 4) while the Wassermann test was almost completely non-reactive.

The treated herd, on the contrary, showed no correspondence between past infection of the animal and reactivity of the test. Thirty-five of the animals had been positive at some time within fifteen months, but all had been negative for from three to eleven months at the time of the tests (with the exception of one animal which was positive at the time the complement fixation tests were run). In one series of 77 serums, where all four tests were run, of 33 serums from formerly infected animals none was positive in all or even in three of the tests, 2 were positive in two tests (precipitin and mercuric chloride), 15 were positive in one test (1 in complement fixation, 1 in adhesion and 13 in mercuric chloride) and 16 were negative, and of 44 serums from animals in which trypanosomes were never found, 4 were positive in two tests (1 in both the adhesion and mercuric chloride and 3 in the precipitin and mercuric chloride), 18 were positive in the mercuric chloride alone and 22 were negative in all four tests (table 10). Additional tests on approximately one-third of the herd furnished corroborative data. On the whole, all of the specific tests (complement fixation, precipitin and adhesion) on the treated herd tended to be non-reactive (tables 7 and 8) whereas the non-specific mercuric chloride test tended to be reactive (table 9), but in neither group could the reactivity or lack of reactivity be correlated with past infection.

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