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The Demonstration and Transmission of the South African Strain of *Trypanosoma equiperdum* of Horses.

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Endeavours made to confirm the tentative diagnosis of Dourine in the early cases by demonstrating the trypanosome were unsuccessful. Sera collected in 1917 from suspected cases of dourine were forwarded to Watson of Canada who established the diagnosis by means of the Complement Fixation Test.

Prior and subsequent to the receipt of Watson's report large numbers of experiments were instituted with an object of demonstrating the parasite and so removing any doubt still present as to the causation of the disease. Walker (1918) who was concerned with the original investigations reported in considerable detail the results obtained by his colleagues and himself. Only once was a trypanosome demonstrated and this one was found in a dog which had been injected with the blood of a horse shewing symptoms of dourine. No success was attained in investigational work carried out between 1918 and 1935 with the object of demonstrating the presence of the trypanosome.

Sera of horses which had received a transfusion of blood from suspected cases of dourine were included in the batch forwarded to Canada. With the Complement Fixation Test these sera also gave positive results. Successful transmission from horse to horse had thus been achieved. Walker did not, however, record the period of time which elapsed between transfusion and collection of the sera. It was possible but most unlikely that sufficient antibody was carried over during transfusion from the positive donor to give a positive reaction to the serum of the recipient. To determine whether such a transference of antibody would occur, 240 c.c. of serum of a positive case, kept in a refrigerator and exposed to 1/100,000 potassium antimony tartrate for 48 hours were injected intravenously into a horse reacting negatively to the Complement Fixation Test on the 3rd and 7th September 1938. The Complement Fixation Test was negative on the 7th and 9th of the same month. On the 14 September five litres of blood were transfused. The test was again negative up to the 10th October. The sera of the horses which had received the transfusion and were reported as positive by Watson were thus true cases of transmission of dourine. In subsequent experiments by Walker and his colleagues the Compement Fixation Test was not apparently employed for the purpose of determining whether transmission of the disease had or had not taken place.

Watson (1920) of Canada in his report provides considerable information on the difficulties he encountered in connection with the transmission and the demonstration of *Trypanosoma equiperdum*. This report will be repeatedly referred to, for a number of the observations made and the difficulties encountered were similar to his. In several instances differences were noted.

Watson states that the disease occurred in a very severe form in Asia and Africa but in an insidious form in America. The disease in the Union of South Africa is also of an insidious type the course at times extending over eight or ten years. The condition of the animals is remarkably well maintained when they are not exposed to bad environmental influences such as insufficiency of food, hard work, intestinal verminosis etc.

In Canada repeated failures to demonstrate the parasite and to obtain transmission in horses and dogs were encountered. In South Africa numerous transmission experiments in horses, dogs and laboratory animals were undertaken during the period 1914-1934. In the experiments reported by Walker 263 animals, of which 63 were horses, were used. For the determination of transmission in the experiments of the above period the laborious and inefficient method of smear examination was employed. If the Complement Fixation Test had been used there is little doubt that positive transmission results would have been obtained, as they were in the few cases when sera were forwarded to Watson.

The collection of a large number of field cases of dourine at Onderstepoort towards the end of 1934 led to the systematic examination of the efficiency of the Complement Fixation Test and ultimately to experiments on transmission in horses. The latter experiments resulted in the demonstration of *T. equiperdum* in the blood of horses and in the transmission of the trypanosomes to equines, dogs and laboratory animals.

Demonstration of T. equiperdum.

Among the positive field cases collected at Onderstepoort was a mare (21103) which was in very poor condition. This animal was killed on 3 May 1935. At autopsy smears were made from various organs and tissues, stained with Giesma and examined. All the smears were negative with the exception of those made from the mammary gland in which a number of trypanosomes were found. The finding of these trypanosomes (by S. W. van Rensburg of this Section) represents the first record of *T. equiperdum* detected in equines in South Africa and placed beyond doubt the diagnosis of the disease.

This early success resulted in the decision to produce recently infected horses for further investigation. Seven mares (21044, 21159, 20722, 21358, 21483, 21176 and 20721) were exposed in a camp to service by a Complement Fixation positive stallion (21400). Of these mares only two (21159 and 20722) did not become positive to the test.

Mare 21483 was killed on 4.3.38 for detailed examination. Smears made from various organs and tissues were negative, but live trypanosomes were found free in the vagina by washing out the vagina with 200 c.c. of saline, centrifuging the wash and examining the deposit as a wet preparation. This was the first demonstration of living *T. equiperdum*.

The above demonstration of living trypanosomes in the vaginal wash led to the examination of the vagina of a live mare (21176), the same technique being employed. Numerous trypanosomes were detected in the wet preparation. Daily blood smears made prior and subsequent to the examination of the vaginal wash were all negative.

Notwithstanding the negative findings of the blood smear examinations it was decided to endeavour to demonstrate the presence of the parasites in the blood of this mare. Watson asserted that it was futile to search for the trypanosomes in the blood. Presumably he meant by means of the examination of blood smears. The technique selected for the search was to centrifuge the plasma obtained by allowing the red cells to sediment out from the citrated blood and then to examine the lowest plasma layers and the deposit which comprised white cells and a few red cells. This method of examination resulted in the findings of quite a number of living trypanosomes in mare 21176. The Tromsdorff graduated tube used for the estimation of the sediment in milk examination is satisfactory for the centrifugation of the plasma. This tube has a capacity of 10 c.c. and a basal graduated projection of 0·2 c.c. which enables the percentage of sediment to be read. By centrifuging the plasma in this tube at 3000 revolutions per minute it was found that the trypanosomes were forced from the 10 c.c. bulk plasma into that in the basal projection and that the lower layers of the plasma in the projection contained more trypanosomes than the upper layers. The wet preparation for microscopic examination was prepared by decanting off and discarding the plasma from the main part of the centrifuge tube when only that plasma and the deposit in the basal projection remained. The upper layers of this remnant were pipetted off and discarded. The remaining plasma in the projection, representing about 0·05 c.c. was mixed with the uppermost portion of the cellular deposit, pipetted off and examined as a wet preparation under a coverslip with a magnification of 300. The concentration of trypanosomes thus obtained was about two hundred-fold.

The trypanosomes became more frequent in the blood as the number of generations in the transmission experiment rose. For the centrifuge method of examination described above there was then substituted a modification which on account of the increased frequency of the parasites proved to be efficient, in most cases, in demonstrating the trypanosomes. The method was to examine under a coverslip a wet preparation made from a drop of the plasma obtained by allowing the red cells to sediment. If no trypanosomes were found by the sedimentation method, the centrifugation method was then used. Ultimately trypanosomes could even be found in some of the cases by the examination of blood smears. There were thus available three methods of examination namely by blood smears, the sedimentation method and the centrifugation method.

Notwithstanding the high concentration obtained by the centrifugation method, it frequently happened that a careful search was necessary to detect any trypanosomes on account of their sparseness. It is thus easily appreciated that the examination of blood smears in such circumstances would be unlikely to give positive results and that the reason for the failure to detect trypanosomes in the blood was not because they were not present in the blood but because they were extremely rare. The technique of both the sedimentation and the centrifugation methods is simple and much less laborious than the examination of a large number of blood smears.

When preparing the wet smears for examination from the plasma and the deposit it is advantageous not to include the red cells and the deeper layers of the white cells for the trypanosomes do not go down, during centrifuging, into the lower layers of the deposit. As the trypanosomes survive in the citrated blood for as long as 72 hours at room temperature, complete sedimentation, which is rapid in equines, should be awaited before pipetting off the plasma for centrifuging.

The examination of the vaginal wash of mare 21176 and other recently infected mares revealed trypanosomes on every occasion. But the examination of the vaginal wash of a number of mares which had been infected for a long time(field cases) gave negative results. The blood of such animals also gave negative results by the centrifugation method. Negative results have also always been obtained in equines which have been retained in the transmission experiment for several months. There is a gradual increase in the circulating blood in the number of trypanosomes which are present in maximum numbers during the second to the fifth week after infection.

The success obtained in the demonstration of trypanosomes in the blood and in the vaginal wash should thus be ascribed to the utilization of recently infected equines in the investigation.

In the cases of dourine available, whether field or artificially produced, no plaques have been seen. Large oedemas have not been uncommon and in stallions scrotal oedemas have been the rule. The examination of such oedemas with one exception has given negative results. The exception was the demonstration of trypanosomes in the oedema of a scrotal sac at autopsy.

Conclusions.

1. The South African strain of *T. equiperdum* was demonstrated in the mammary gland, in scrotal oedema and in the vagina at autopsy, and in the vagina and the blood of living horses.

2. Improved methods of microscopic diagnosis for the detection of trypanosomes are described.

Transmission of Dourine in Horses, Mules and Donkeys by Blood Inoculation.

Experiments on the artificial transmission in horses of dourine in South Africa by the injection of blood, serum, oedematous fluid, washings of the uterus and vagina, spinal fluid and of macerated tissues had all been unsuccessful. Watson (1920) failed to demonstrate the parasites in a large number of horses and dogs inoculated with blood and other tissues before he succeeded in 1907 in finding trypanosomes in certain typical lesions of an inoculated animal. He did not use blood for this inoculation. He states that only during a very active stage of the disease as during a period of plaque eruption is transmission likely to be successful and that, otherwise, direct transfusions of blood or the inoculation of large amounts of blood at intervals over several weeks failed to set up infection. In 24 essays in horses he did not succeed in finding trypanosomes in the blood in a single case.

The finding of the causal agent in the blood of a mare infected by coitus considerably increased the chances of a successful transmission by the inoculation of blood, even though the number of parasites in the blood was so small as to necessitate the use of the centrifugation method to demonstrate their presence. Frequent blood smears made from this mare were negative.

Prior to the commencement of these blood transmission experiments, a transmission by means of a vaginal wash inoculation was carried out. To determine whether transmission had or had not occurred it was necessary to rely on the Complement Fixation Test as the blood concentration methods had not then been devised. A mare which was negative to the Complement Fixation Test was inoculated with a vaginal wash containing living trypanosomes. The injections were made onto and under the vaginal mucosa and into the vagina. The test became positive and remained so until the death of the animal.

The disease produced by the injection into the undamaged vagina of a mare of the strain of *T. equiperdum* used for the production of antigen was of an acute type, the trypanosomes in the blood smears and the elevation of the temperature appearing on the 7th day.

On the day (20 October 1938) that trypanosomes were demonstrated in the blood of the circulation by the centrifugation method of examination in the mare 21176, one of the group infected by coitus, one litre of its blood was transfused into a gelding (21470) which was negative to the Complement Fixation Test. Trypanosomes were found in the blood of this gelding on the 21 November 1938, by means of the centrifugation method of examination. The blood smears made from this animal were negative.

Thus the first essay in the transmission of the South African strain of *T. equiperdum* from horse to horse by the inoculation of blood known to be positive was successful. The donor of the blood at the time of inoculation did not shew symptoms of the disease, plaque or oedemas were not present, the habitus was good, the temperature was not elevated and the Complement Fixation Test was negative.

The trypanosomes with which this transmission was attained were subsequently maintained in horses, mules and donkeys for 4 years through 24 generations. No difficulty was experienced and no failure was encountered in the transmission even though the amount of citrated blood injected intrajugularly was, at times, as small as 50 c.c. and the intervals between the injections sometimes were as long as 4 months. Frequently negative examinations of the blood of some of the animals by means of the centrifuge method were obtained at the time of the subinoculations but, nevertheless, transmissions were always successful.

It was not until the 4th generation that trypanosomes were found in the blood smears prepared from the animals (31 January 1939). In the early generations when the number of parasites in the blood was extremely small, even the centrifuge method of examination was, at times, negative, and the positive findings by this method were only present for a week or two. As the number of generations increased, so did the number of parasites in the blood become more frequent. Ultimately the sedimentation method of examination and even at times the blood smear examination could be used in most cases with satisfactory results. Furthermore the trypanosomes were detectable over a much longer period in the various individuals.

The virulence of the strain has not been markedly increased by the above passage, the reaction in the animals being still mild and the course long. A temperature and blood examination chart of donkey 21777 (23rd generation) is included to illustrate this. (Chart 1.) In all cases of transmission the animals were adults.

The periods between the generations, the animals used and some details of the examination are tabulated in the table. From this it will be seen that the periods between the generations varied considerably and that the blood smear examinations were of no diagnostic value in the early generations. Horses, mules, and donkeys were used in the transmission experiments.

Conclusions.

1. No difficulty was experienced in the transmission of the South African strain of *T. equiperdum* by means of the inoculation of the blood of a mare in which trypanosomes had been demonstrated.

2. The blood concentration methods of examination were much more efficient and less laborious than the blood smear examinations and it was due to these methods that the transmission experiments were successfully completed. Failures in previous experiments on transmission were apparently due to inefficiency in diagnosis.

3. The number of trypanosomes in the blood increased as the number of generations increased but the parasites could be detected in the individual animals over only a short period of the course of the disease.

TRANSMISSION OF T. EQUIPERDUM BY BLOOD INOCULATION.

	Number.	Dates of sub-inoculation.	Remarks.
—.....	21176	---	Infected by coitus. *T. equiperdum* in centrifuged blood.
1.....	21470	20/10/38	*T. equiperdum* in centrifuged blood: blood smears negative.
2.....	20477	24/11/38	Blood smears negative.
3.....	21667	8/12/38	Blood smears negative.
4.....	16660	14/1/39	Blood smears positive.
5.....	21908	10/2/39	,, ,, ,,
6.....	21657	23/2/39	,, ,, ,,
7.....	21658	12/4/39	,, ,, ,,
8.....	20721	16/5/39	,, ,, ,,
9.....	20962	8/8/39	,, ,, ,,
10.....	20137	25/10/39	,, ,, ,,
11.....	21226	4/3/40	,, ,, ,,
12.....	22237	18/3/40	,, ,, ,,
13.....	22239	30/3/40	,, ,, ,,
14.....	22242	10/4/40	,, ,, ,,
15.....	22244	4/5/40	,, ,, ,,
16.....	22286	6/6/40	,, ,, ,,
17.....	22349	6/8/40	,, ,, ,,
18.....	22344	24/10/40	,, ,, ,,
19.....	22088	14/3/41	,, ,, ,,
20.....	22257	3/5/41	,, ,, ,,
21.....	21293	16/9/41	,, ,, ,,
22.....	22161	12/10/41	,, ,, ,,
23.....	21777	21/2/42	,, ,, ,,
24.....	21946	24/7/42	,, ,, ,,

Transmission of the South African Strain of *T. equiperdum* to Animals other than Equines.

Once transmission in equines by blood inoculation was attained it was thought that little or no difficulty would be experienced in the transmission to other animals. The contrary, however, was the case. Sub-inoculation of citrated blood of horses containing trypanosomes into dogs and rabbits did give positive results as judged by blood smear examination but the passage was not successful after the second generation.

This question is dealt with in another article of this series.

A Comparison of the South African and the Antigen Strains of *T. equiperdum*.

The South African strain of *T. equiperdum* utilized was the one derived from horse 21176 in the course of the transmission experiments. The antigen strain was imported from Europe in 1921, and maintained, since then, in guinea pigs for the purpose of production of antigen for the Complement Fixation Test. A third strain utilized was the South African strain transmitted to rats and maintained in them for 35 generations.

No difficulty was experienced in the transmission of the antigen strain from guinea pigs to equines. The infection resulting from the subcutaneous injection of the infected guinea pig blood or the infusion of such blood into the vagina was an acute one, the equine shewing a rise in temperature and trypanosomes in the blood smears within a few days, a rapid development of anaemia and a marked loss of condition (20529 Chart 2).

When the injection of the antigen strain was made into a natural South African Complement Fixation positive case of dourine, the response obtained was very similar to that obtained by inoculation into an equine not infected with dourine. Again the temperature elevation, the development of anaemia, the early appearance of parasites in blood smears, and the rapid loss of condition were conspicuous features. (20935, Chart 3 and 20936 Chart 4).

A similar response was obtained by the injection of the antigen strain into an equine which had been infected (22nd generation) by the inoculation of the South African equine strain. (22161, Chart 5).

And finally the injection of the antigen strain (21293 Chart 7) into an equine which had previously been infected with the South African equine strain (21st generation) and the South African strain maintained in rats for 35 generations produced a very similar response to that obtained in a control animal.

Every equine injected with the antigen strain died unless treatment was instituted.

When the South African strain passaged in rats for 35 generations was used as the inoculum in equines of the transmission experiment i.e. equines which had been injected with the South African strain, no marked response was obtained (Chart 6). In a fully susceptible negative animal the response to this strain was only slightly more in evidence than when transmission was from equine to equine. In the latter case a severe reaction was never produced even after many generations of passage. There is little or no elevation of temperature, anaemia development is absent or very

slight, the trypanosomes are rare in the blood, many blood smears being negative, loss of condition is not marked and death never results. The repeated transmission from equine to equine has resulted in an appreciable increase of the number of trypanosomes in the blood but not in a marked increase of virulence.

Conclusions.

From the above experimental inoculations it would appear that the maintenance of *T. equiperdum* for 24 generations in equines has not resulted in an appreciable increase of virulence, that the maintenance through 35 generations of rats has slightly increased the virulence and that the European strain shews either marked differences in comparison with the South African strain or a marked increase of virulence due, possibly, to its maintenance in guinea pigs for more than 20 years.

THE RESPONSE OBTAINED BY THE INOCULATION OF THE SOUTH AFRICAN STRAIN INTO HORSES GIVING DOUBTFUL RESULTS WITH THE COMPLEMENT FIXATION TEST.

During the course of an experiment devised for the purpose of studying the efficacy of the Complement Fixation Test, it was found that certain horses gave results which were either positive or doubtful or negative at different tests which were usually at monthly intervals. The doubtful or negative results were obtained whenever the antigen employed was not up to standard, notwithstanding that the majority of horses under test gave clear-cut positive results. Positive results were also always obtained with the sera of *Trypanosoma brucei* cases. The equines giving these variable results to the test were, for convenience, described as "low antibody" cases. "High antibody" cases were those which gave positive results to the test even when the antigen was not up to standard.

When the South African strain was inoculated into the "high antibody" cases no response was obtained. Trypanosomes could not be detected in the blood even when the concentration methods of examination were utilized.

But the inoculation of the South African strain into the "low antibody" cases did produce a response, trypanosomes appearing in the blood. However, the parasites were in smaller numbers and detectable over a shorter period than in negative controls inoculated at the same time and with similar amounts of infected blood.

As the result of the experiments it is a justifiable conclusion that these "low antibody" cases are not true cases of dourine, notwithstanding that they do, at times, react positively to the Complement Fixation Test. If such a conclusion be accepted, it means that a certain number of horses comparatively few probably, are diagnosed by means of the Complement Fixation Test as dourine when actually negative. The conclusion that such cases were not actually dourine was supported by the fact that a negative stallion which was utilized to serve, during two consecutive seasons, five of these "low antibody" mares, did not contract the disease.

Although the inoculation of the South African strain was utilized as above to determine whether a horse under test was or was not a true case of dourine, this procedure, obviously, could only be of value in cases submitted for special examination. As an example of such an application

may be mentioned the case of two mules which reacted positively to the Complement Fixation Test in an area regarded as being free of the disease. The two mules together with, as control, one susceptible mule and one " low-antibody " horse were all injected with the South African strain. The examination of the blood by the centrifugation method consisted of 10 examinations over 24 days, and the results obtained were for the susceptible mule ten positives, for the " low antibody " horses three positives and for the two mules reacting positively to the Complement Fixation Test, four positives and no positives respectively. Of the two mules under test one, thus, gave a result comparable with that obtained in the " low antibody " case i.e. it was not a case of dourine, whereas the other was a case of dourine.

It may be mentioned here that the improvement of the technique of the Complement Fixation Test resulting from observations made during the past ten years has been such, that the " low antibody " cases i.e. cases which formerly gave with the less efficient test positive, doubtful, or negative reactions at various tests, now always give positive reactions. There thus occurs in routine testing of large numbers of equines a certain number, admittedly small, which with antigen prepared with special care, give positive reactions notwithstanding they are truly negative. Separation of these from true positives can, however, be achieved by instituting controlled decreased efficiency of the test. This point is dealt with in another chapter of this series.

Conclusions.

It is possible to determine by the inoculation of the South African strain of *T. equiperdum* and the subsequent examination of the blood for parasites whether such cases are or are not true cases of dourine.

Discussion.

The identification of *Trypanosoma equiperdum* was delayed for more than 20 years in South Africa for the reason, apparently, that the very inefficient method of blood smear examinations was utilized for the purpose of diagnosis of the disease in natural cases, many of which were undoubtedly in advanced stages of the disease. It is only during the first few months of the course of the disease that the blood examination enables a diagnosis to be made. The most efficient method of examination of the blood for the parasites is by centrifuging the plasma and examining the deposit thus obtained. The microscopic examination of the wet preparation prepared from the plasma after sedimentation of the red cells also gives results greatly superior to those obtained by stained blood smear examination.

The parasites are only found with ease by the above methods of examination for a comparatively short period of the course of the disease, notwithstanding that the animals remain positive to the Complement Fixation Test. Horses affected with South African dourine may live for many years without shewing any obvious indications of having the disease provided that the environmental conditions are not of a severe nature.

When parasites cannot be detected by the above mentioned means, the Complement Fixation Test serves excellently for the purpose of determining whether the case is positive or not. A disadvantage of the test, however, is that it does not usually give positive reactions until about 30 days after infection whereas parasites may be detected in the blood in about 10 days.

The transmission of the South African strain of *T. equiperdum* in horses (mares, geldings, and stallions), mules and donkeys by the intravenous injection of infected blood presented no difficulties. Diagnosis was in every case made by the identification of the parasites in the blood of the inoculated animal. Much more difficult was the transmission of the South African strain of *T. equiperdum* from equines into other species, and the injection into animals such as dogs, rabbits and guinea pigs for the object of arriving at a diagnosis is definitely of no value.

The difference between the South African strain maintained in equines and the same strain maintained in rats for 35 generations is small whereas the difference between these and the European strain maintained in guinea pigs and utilized for antigen production is very considerable.

The injection of the South African strain into cases which during the course of routine examination are classed, at tests made at different times, as positive, doubtful or negative, gives results which assist in determining whether such are or are not true cases of dourine.

Summary.

The identification and the transmission of the causal trypanosomes of the South African type of Dourine has resulted in an improvement of the methods of diagnosis by means of the examination of the blood and of the Complement Fixation Test.

My thanks are due to Dr. P. J. du Toit, the Director of Veterinary Services, for the facilities placed at my disposal, to Dr. G. de Kock, the Deputy Director, for his valued advice and encouragement and to Dr. Robinson, the Sub-Director, for all the Complement Fixation Tests.

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CHART 1.

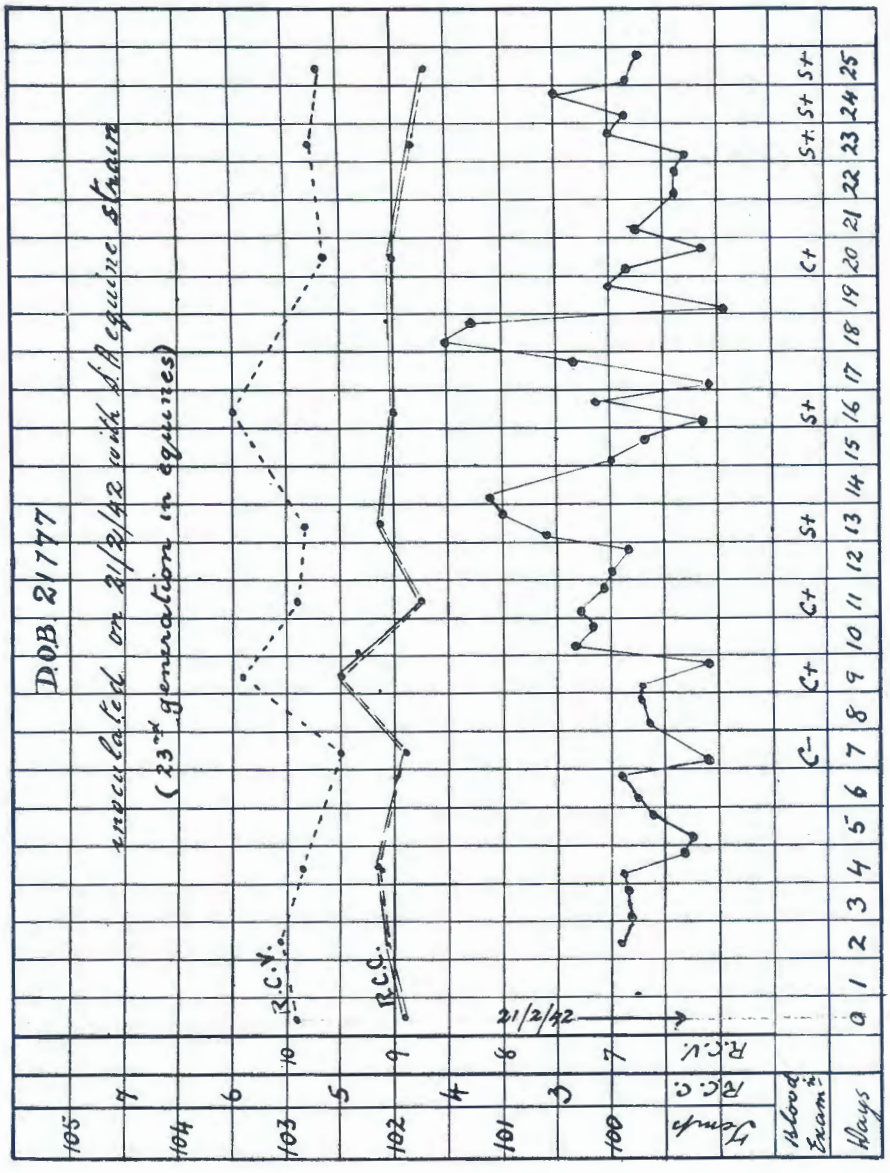

D.O.B. 21/7/7
inoculated on 21/2/42 with A.B. equine strain
(23rd generation in equines)
R.C.V.
R.C.C.
21/2/42
Days
Blood Exam.
Temp.
R.C.C.
R.C.V.
C− = Negative by centrifugation.
C+ = Positive by centrifugation.
S+ = Positive by sedimentation.

CHART 2.

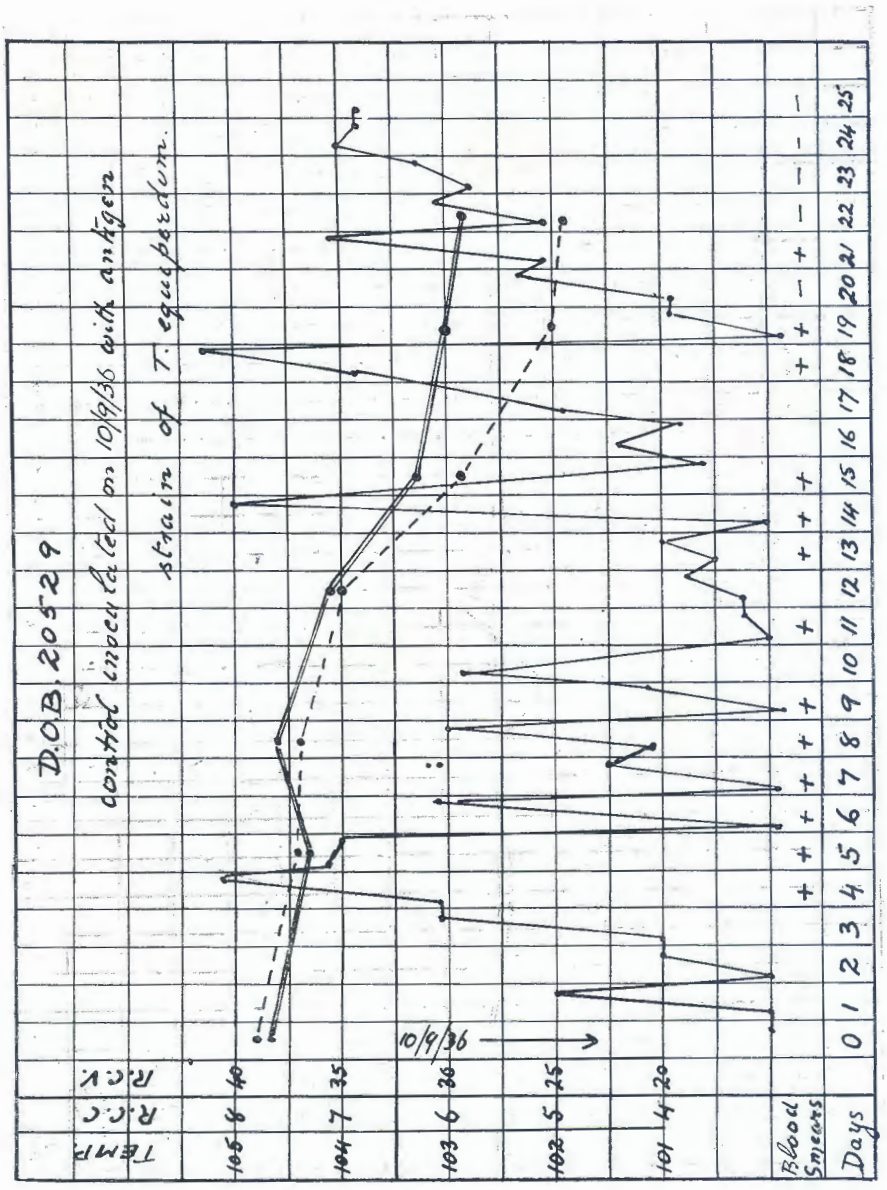
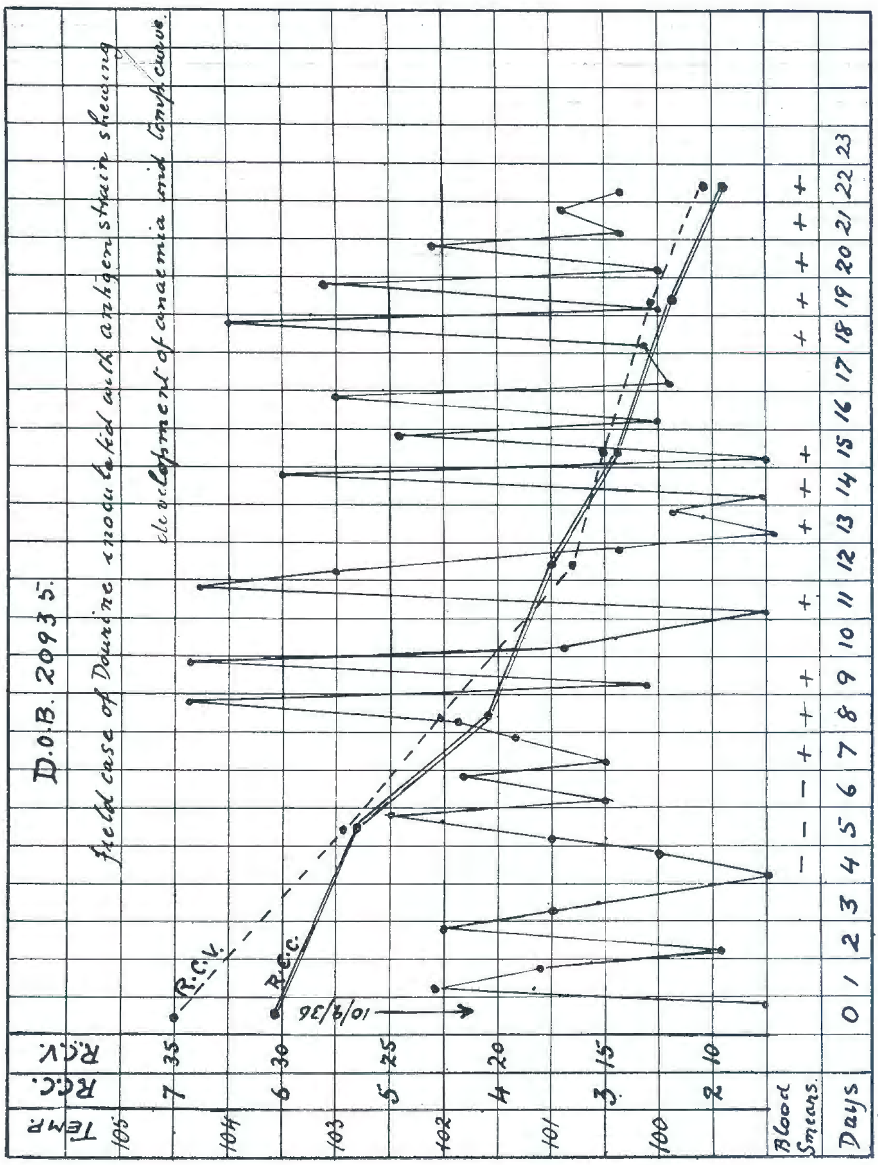
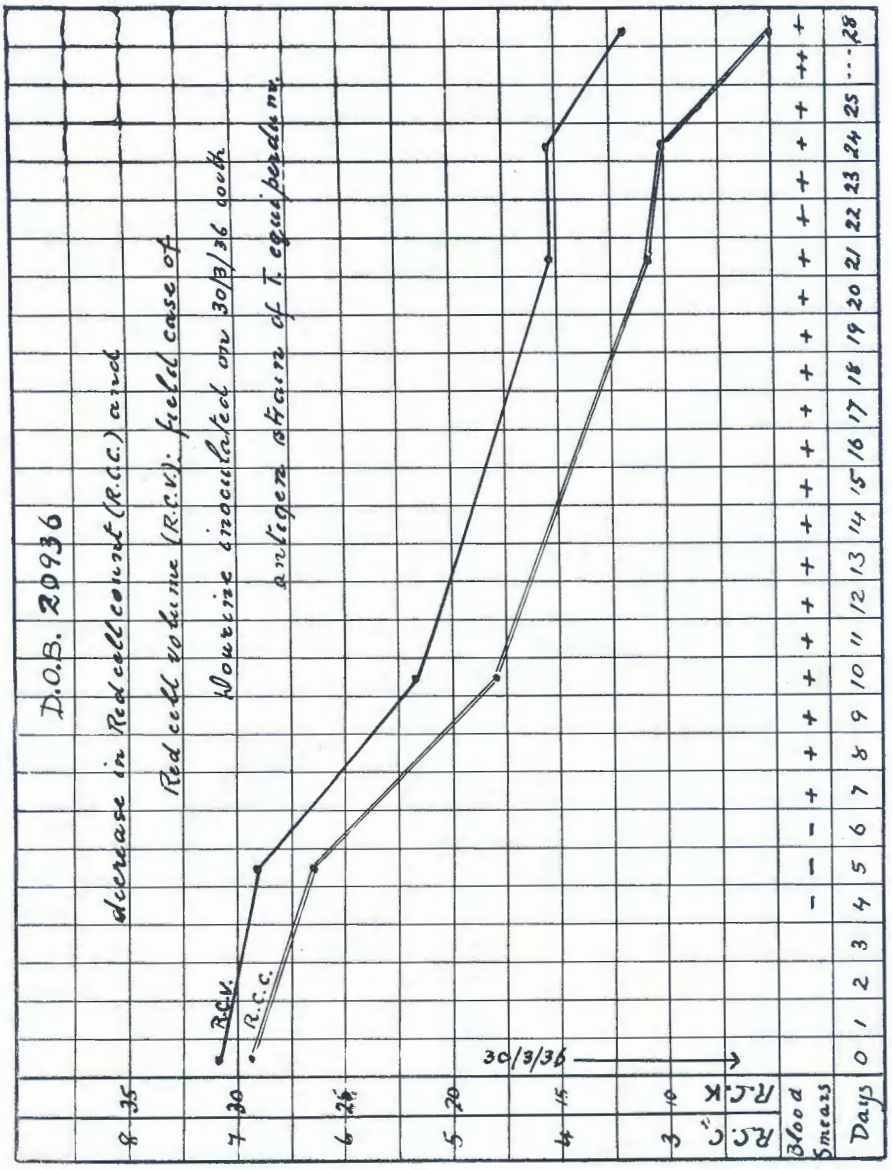

D.O.B. 20-5-29
control inoculated on 10/9/36 with antigen
strain of T. equiperdum.
TEMP
R.C.C
R.C.V
105 6 40
104 7 35
103 6 30
102 5 25
101 4 20
10/9/36
Blood Smears
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
+ = Positive blood smear.
− = Negative blood smear.

CHART 3.



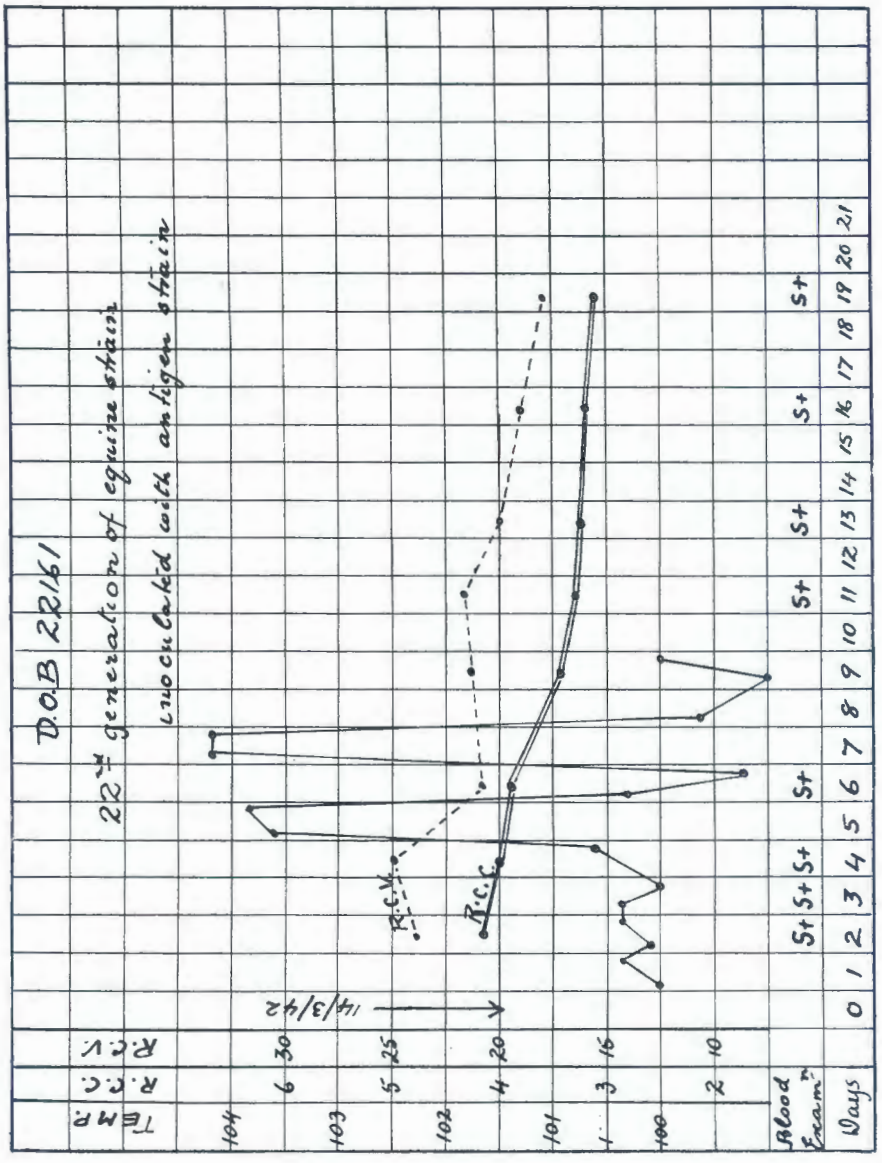
+ = Positive blood smear.
− = Negative blood smear.

CHART 4.



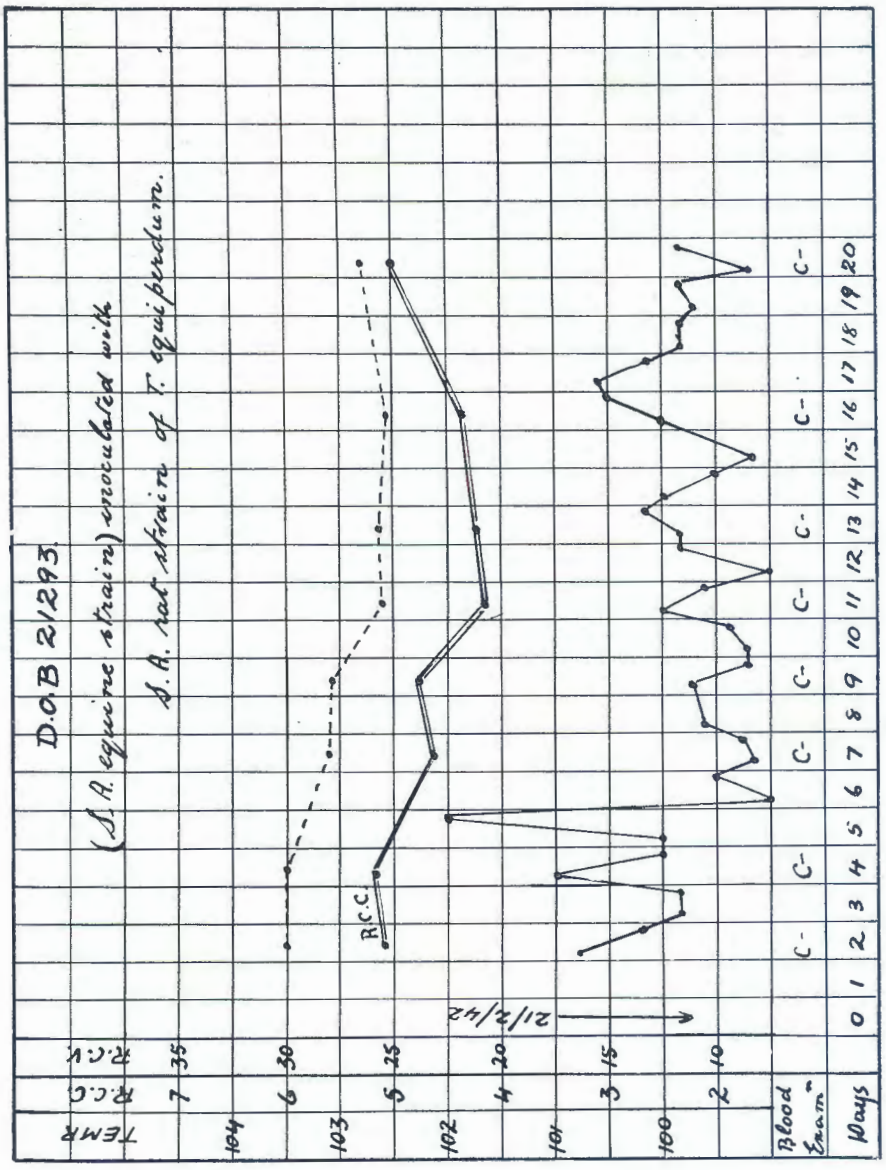
+ = Positive blood smear.
− = Negative blood smear.

CHART 5.



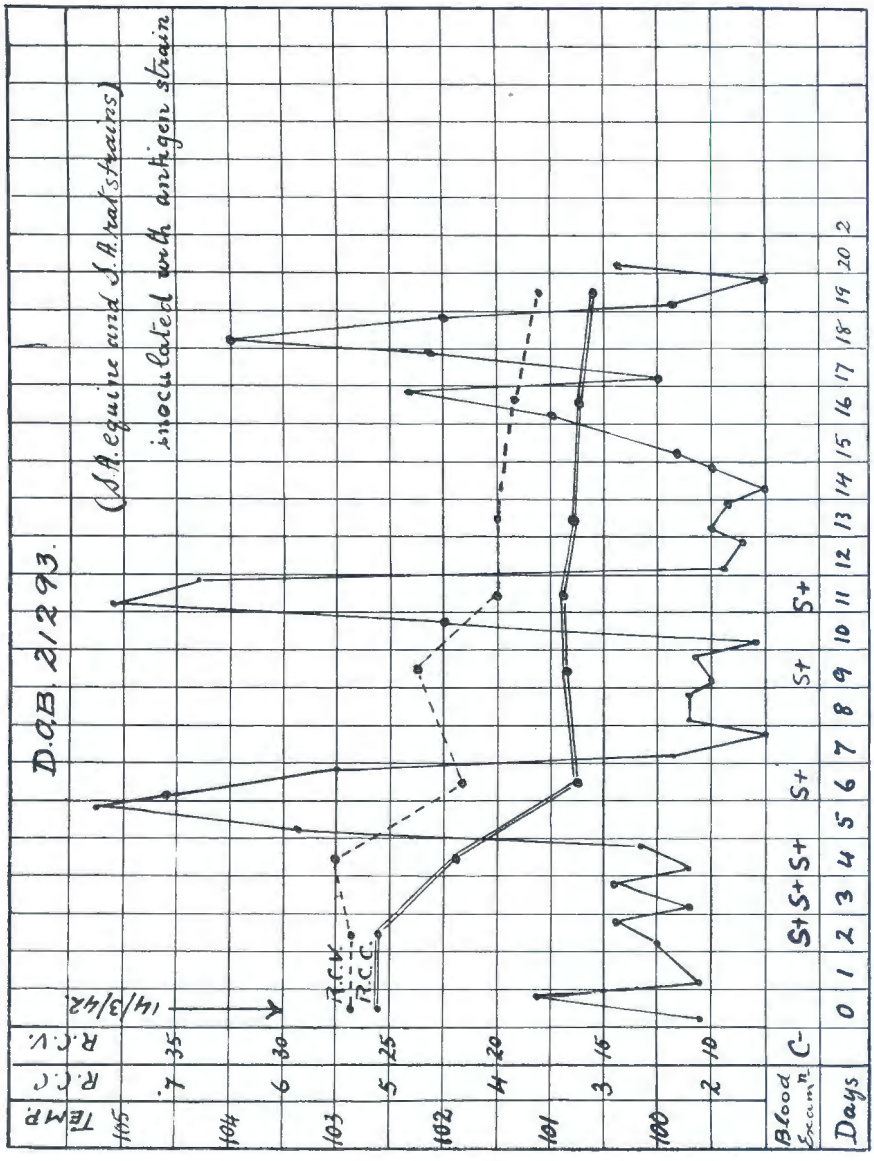
S+ = Positive by sedimentation.

CHART 6.



C– = Negative by centrifugation.

CHART 7.



C− =Negative by centrifugation.
S+ =Positive by sedimentation.