On the value of the different bacteriological methods of diagnosis of typhoid fever / by Sheridan Delépine.

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ON THE VALUE

OF THE

DIFFERENT BACTERIOLOGICAL METHODS OF DIAGNOSIS OF TYPHOID FEVER.*

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It is generally acknowledged that in a large number of cases which may, on good grounds, be suspected to be cases of typhoid fever, it is by no means easy to establish, by ordinary clinical methods, a positive or a negative diagnosis leaving no room for doubt.

For several years I have paid much attention to the methods which bacteriology has placed at our disposal, and which are capable of being advantageously used for the diagnosis of infectious diseases. On the accuracy of the diagnosis of such diseases depends entirely the value of our statistics and of the methods we use in the treatment and prevention of disease. I have done this not only with the object of satisfying myself that these methods were useful, but more especially with the view of discovering how far they were capable of giving more reliable results than other methods within a space of time and under conditions which rendered their application advantageous. It was also in every case necessary to discover the technique which reduced to a minimum the errors almost necessarily connected with biological investigations. I have, on previous occasions, had opportunities to give an account of work of this kind in connection with tuberculosis, Asiatic cholera, and diphtheria; this time I will refer more specially to the diagnosis of enteric fever.

Previous to Eberth (1880—83) and Gaffky (1884) several observers had described micro-organisms in connection with enteric fever (von Recklinghausen, Klein, Klebs, among others).

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Since Gaffky, the intimate relations existing between typhoid fever and the bacillus discovered by Eberth, have been more and more clearly recognised.

Gaffky and observers who have followed him have shown that it is almost always possible to obtain the bacillus typhosus from the spleen or mesenteric ganglia of patients who have died of typhoid fever. This bacillus cannot be obtained from the organs of patients who have died of other diseases.

It is necessary, in order to obtain that result, not to wait till post-mortem changes have allowed the penetration of the common intestinal bacteria (and more specially of the bacillus coli communis) into the viscera. This penetration does not take place very rapidly when the external temperature is not high. To test this point I have examined bacteriologically the spleen of five patients who had died of various forms of malignant tumours, from twelve to twenty-four hours after death (the external temperature being under 16° C. in all cases), and have found micro-organisms cultivable in only two of these cases. In one of them the bacillus coli was the only organism present; in the other cocci were found.

In five cases of patients who had died of typhoid fever during the fastigium, and whose spleen was found more or less enlarged and typical (post mortem), I have been able to obtain from this organ a bacillus giving all the reactions which we now associate with the bacillus typhosus. In two of these cases I found, in addition, the bacillus coli.

But though it is easy for any one familiar with ordinary bacteriological methods to prove the presence of the typhoid bacillus in the organs of patients who have died of typhoid fever at an early stage, it is, on the contrary, difficult to isolate that microbe from the secretions or other products of a patient during life. In saying so, I presume that puncture of the spleen will not be considered a justifiable procedure.

This difficulty is due to the fact that the bacillus of Eberth grows more slowly and is more easily killed than some of the organisms which are found associated with it in dejecta.

I think it was in 1885 that the bacillus of typhoid fever was first described as occurring in the stools of typhoid patients. The same year Escherich demonstrated that in the intestine of milk-fed infants there was a bacillus which resembled closely the bacillus of typhoid fever. This bacillus—coli communis, as we know it now—is a constant inhabitant of the intestine of man and of other animals, and though, under certain circumstances, it shows itself to be exceedingly pathogenic, it does not usually indicate any diseased state.

Yet it must be remembered that, like the typhoid bacillus, it may pass during life and after death beyond the walls of the alimentary

canal; that it may be found in the spleen, in the peritoneum, etc., and that, like the typhoid bacillus, it may pass through the kidney and be found in albuminous urines. In a very interesting case which I had the opportunity of studying two years ago with Professor Dixon Mann, the kidneys excreted such a large number of these bacilli that the urine even within the bladder was rendered, for several days before death, very distinctly turbid.

But it is not only within the body, during life or after death, that this bacillus coli communis may be found. It is for obvious reasons abundant in ordinary sewage and in water much contaminated with sewage. Indirectly, milk and other products may also get infected.

Within the last few weeks I have examined, among a number of others, two samples of milk, apparently quite fresh, and such as are found usually on the market. When small doses (2 ccm. to 5 ccm.) of these milks were injected under the skin or into the abdomen they caused the death of the animals inoculated within forty-eight hours. From the abscesses produced and from the peritoneal exudations I have cultivated a bacillus resembling closely the bacillus of typhoid fever, but which on being submitted to various tests proved to be the bacillus coli communis. In 1894 I found a large number of the same bacilli in milk which had produced a serious outbreak of diarrhœa in Manchester. The investigations carried out by Dr. Niven at the time proved that the milk had been exposed to gross contaminations. (It is interesting to note that the conclusion that such must have been the case had been indicated by the bacteriological examination before the farm had been inspected.)

The bacillus coli may therefore pass from the human body into surrounding media, and be discovered in water, soil, dusts, milk, etc., where, without proper care, it may be mistaken for the bacillus of typhoid fever. This has undoubtedly been formerly a serious source of error.

The bacillus coli communis can now be clearly differentiated from the bacillus typhosus by appropriate tests, and no great difficulty would arise from the resemblance of the two bacilli if other bacilli, resembling in some respects both, were not present in external media and more specially water. Weichselbaum, Cassedabat, and others have described many of these bacilli. I have myself found several such bacilli in Manchester water. It has become customary among bacteriologists, owing to these facts, to speak no more of a bacillus coli, but of a group of bacilli, called coliform bacilli, or "bacilli of the coli type," whenever the bacilli referred to have not been submitted to all the tests which are necessary to identify Escherich's bacillus.

The diagnosis of the bacillus of typhoid fever cannot be based safely at the present time on any character of growth on ordinary media, for

there is hardly any one of these characters which cannot be reproduced singly or in groups by some of the spurious bacilli. It becomes therefore necessary to use a series of characters which exclude all other organisms which might be mistaken for the typhoid bacillus. This difficulty is so well acknowledged that in 1895 Lösener* gave at least 11 characteristic signs by which to recognise the bacillus typhosus :—

(1) Characteristic appearance of gelatine cultures. (2) Great motility—variation of forms on suitable media. (3) Great number of cilia. (4) Non-colouration by Gram's method. (5) Non-production of gas in media to which glucose, lactose, or saccharose have been added. (6) Non-coagulation of milk. (7) Absence of indol in cultures. (8) Acid reaction of cultures made in neutral whey not more than 3 per cent, as estimated by decinormal solution of sodium hydrate. (9) Culture on suitable potato : transparent glaze. (10) Slow growth in normal solution of Maasse, to which glycerine has been added. (11) Pathogenic action.

I have myself, during the last few years, tried most of these tests, and many others not mentioned in the above list, as they became known, and I have gradually been led to consider a number of them quite unnecessary, because they gave no better results than simpler methods. On the other hand, by repeated examination of bacilli isolated from perfectly clear cases, and which could be considered as typical, I have been led to adopt the following tests as giving good results with the bacillus typhosus and the bacillus coli, either immediately after the bacilli have been isolated from organs or morbid products, or after they have been kept for some years in the laboratory. Yet, as most of these tests may be described as negative with regard to the Eberth's bacillus, and positive with regard to the bacillus of Escherich, and as the latter may occasionally fail to display its usual activity in some media, it is difficult to base a diagnosis on any of the following characters, taken singly, and a perfect diagnosis must be based on the complete series of reactions given on the opposite page.

It will be noticed that I do not recommend now the use of media containing antiseptic substances, such as carbolic acid, hydrochloric acid, formalin, etc. These I have all tried, and found to be of little use either for differentiating the bacillus coli communis from the typhosus or for separating these bacilli from various products containing other bacteria. I rely entirely, and have done so for several years, in my own work on dilution of media. By reducing the number of colonies, obtained in a plate, to five, ten, or twenty at most, I can watch the development of these colonies sufficiently long to separate the bacillus of typhoid fever from other bacilli. The advantage of this method is that the growth of the typhoid bacillus is much more rapid on

* *Arbeiten aus dem Kaiserl. Gesundheitsamte*, Band XI.

TABLE SHOWING THE MODE OF GROWTH AND THE EFFECTS OF CULTIVATION OF THE BACILLUS TYPHOSUS AND THE BACILLUS COLI COMMUNIS ON VARIOUS MEDIA.

Media.	Bacillus Coli Communis.	Bacillus Typhosus.
1. Alkaline peptone, bouillon gelatine. Alk. = carbonate sodium, 2 : 1000, ·22° C.	Growth rapid — superficial colonies often thick—more opaque and more coarsely granular than in the case of the bacillus typhosus (24 to 48 hours).	Growth distinctly slower—superficial colonies usually very thin, transparent, with deeply indented border, and surface marked by wavy bundles (48 hours).
2. Slightly acid potato gelatine,* 22° C.	ditto.	Growth very slow; colonies remain small and transparent.
3. Slightly acid potato (tested by growth of a typical typhoid bacillus), 37° C.	Rapid abundant growth, pale or dark brownish yellow (24 to 48 hours).	Transparent, very thin glaze-like growth (24 to 48 hours).
4. Glucose gelatine, stab. or diffuse culture, 22° C.	Abundant production of gas bubbles (24 to 48 hours).	No production of gas.
5. Alkaline peptone bouillon, 37°.	Production of indol, variable in quantity (48 hours).	No production of indol.
6. Feebly alkaline, litmus, lactose agar peptone bouillon, 37° C.	Production of acid (litmus becomes bright red) (24 to 48 hours).	Litmus never turns red.
7. Separated sterilised milk, 37°.	Coagulated (24 to 48 hours).	Never coagulated.
To these tests I now add the agglomeration reaction.		
8. Recent (20 hours) bouillon culture and 1 part in 10 of blood or blood serum from a typhoid patient, examined microscopically.	Usually no stoppage of motion and no agglomeration within 1 hour.	Variable amounts of immobilisation and agglomeration usually well marked within 5 minutes.

* The addition of iodide of potassium suggested by Elsner has not seemed to me to present any distinct advantage.

CHARACTERS OF THE BACILLI WHICH CAN BE USED IN CONNECTION WITH THE CULTIVATION TESTS.

	Bacillus Coli Communis.	Bacillus Typhosus.
1. Size.	Variable, specially in cultures. Diameter, 0·3 to 0·6 micros.	Variable; there are always some larger and thicker bacilli among the others 0·7 to 0·9 micros.
2. Number of cilia.	Maximum, 6. Not easy to stain.	10 to 12. Easy to stain by Van Ermengem's method.
3. Staining reaction.	Not well stained by Gram's method. Stains well by alkaline methylene blue (Loeffler); *id.* carbol-fuchsin (Ziehl).	

ordinary alkaline media than on those to which antiseptic substances have been added for the purpose of checking the growth of other bacteria. On such alkaline media it is possible to obtain in twenty-four hours colonies of typhoid bacilli of such a size that it would take three or four days to grow on acid, carbolised, or iodised gelatine.

Dr. Carver, who has of late been examining, at Owens College, a large number of specimens of products from typhoid patients, has almost given up Elsner's method after trying it for a long time, and uses now the dilution method which I recommend as being both simpler and more effective. He found, also, that it is not easy to isolate from typhoid stools a bacillus giving all the reactions mentioned above.

In order to determine accurately the advantages and disadvantages of the use of antiseptic substances in cultivation media, I asked Dr. Richmond, who was working in my laboratory during 1894, to make a number of observations on the mode of growth of the bacillus coli and bacillus typhosus after the addition of various proportions of hydrochloric acid, carbolic acid, and formaldehyde. He found that invariably all these substances delayed considerably the growth of both bacilli when their amount was sufficient to interfere with the growth of the liquefying organisms found in the fæces. He found, also, that when the proportion of carbolic acid reached 0·27 per cent neither the bacillus coli nor the bacillus typhosus would grow. When the proportion was 0·23 per cent the bacillus coli alone could grow; the bacillus typhosus was able to develop only when the proportion was reduced to less than 0·23 per cent, and under these conditions the bacillus coli grew much more readily than the bacillus typhosus.

In order to see whether some of the differences observed between the two bacilli were not due to a certain extent to passage through the tissues of the patient, I asked Dr. Ainsworth to study specially the reactions of the bacillus of typhoid fever and of the bacillus coli immediately after their separation from the spleen of some patients who had died from typhoid fever. In a series of cases which he examined with great care the reactions of both bacilli were absolutely typical from the first. His investigations were, however, unfortunately interrupted before they were completed, and they have not been published.

Dr. Carver, who is working at the same subject from another point of view, has found in the fæces of typhoid patients bacilli resembling closely the bacillus of typhoid fever, but which, on being submitted to the series of tests given above, usually produced one or two of the reactions typical of the bacillus coli.

From the enlarged spleen and mesenteric glands of undoubted cases of typhoid fever I have, on the contrary, never failed to obtain pure cultures of a bacillus giving, without exception, all the reactions of the typhoid bacillus.

The conclusions from all this are, it seems to me, very clear :—

(1) It is easy to prove the presence of the typhoid bacillus in cases of typhoid fever if one can get access to such internal organs as the spleen and mesenteric glands in the first weeks of the fever and before post-mortem decomposition has set in.

(2) It is not easy to separate the bacillus typhosus during life, unless one obtains blood from the spleen (which is not to be recommended), or from a rose spot. The separation of the bacillus from the stools and urine is attended with serious difficulties.

(3) The separation of the bacillus in the most favourable cases takes at least twenty-four hours, and the testing of pure cultures of the suspected bacillus by the ordinary methods takes from twenty-four to forty-eight hours, so that a perfectly reliable diagnosis of the bacillus typhosus cannot be made by the methods known till the beginning of last year in less than from two to three days, but under ordinary circumstances such a diagnosis would take three or four days.

A first improvement in this state of things was due to the discovery made by Pfeiffer* of a specific reaction which occurs when the vibrio of cholera is injected into the peritoneal cavity of a guinea-pig immunised against cholera. This "Pfeiffer's reaction" was found afterwards by Pfeiffer and others to be applicable to other micro-organisms than the microbe of cholera, and more especially to the bacillus of typhoid fever and to the bacillus coli communis.

Metchnikoff and Bordet showed that it was not necessary to inject the micro-organisms into the peritoneal cavity of a living animal in order to demonstrate the specific reaction ; it was enough to add the fresh serum of an immunised animal to the microbes "in vitro."

Gruber and Durham studied more specially the reaction exhibited by various micro-organisms when brought in contact with the blood serum of animals severally immunised against each of these microbes. They confirmed the specificness of the reaction within certain limits carefully indicated by them. They agreed with Metchnikoff and Bordet in showing that the reaction observed "in vitro" is as specific as Pfeiffer's reaction observed "in vivo," and they established, by careful experiments, the conditions necessary to obtain a reaction sufficiently specific to be of diagnostic value when the special technique which they adopt is followed.

With special reference to diagnosis, the outcome of the work of these observers was that, when a bacillus had been isolated from pathological discharges or external media, and one wished to establish its identity, it had become possible to do so within one hour by means of the blood

* Pfeiffer (R.).—*Zeitschrift für Hygiene*, 1894, XVIII., p. 1; 1895, XIX., p. 194. Pfeiffer (R.) and Kolle, 1896, XXI., p. 203.

serum of an immunised animal. But it was necessary first to separate the bacillus which had to be tested, and, as we have seen, this is not always easy or possible.

Still *speaking from the diagnostic point of view*, the next great advance was that made by Widal. This does away entirely with all the difficulties connected with the separation of the micro-organism during the life of the patient. In this way the time necessary to make the diagnosis is much shortened, and the diagnosis is made more certain also, for reasons which cannot be entered into here.

Whilst Widal was working in Paris at this improvement, work was also being done by Grünbaum, in Gruber's laboratory. Widal, however, had given a complete account of his results, and these had been fully confirmed by others, before Grünbaum's results appeared in the papers. With the exception of a suitable reference to Grünbaum's work, with which I was unacquainted in September last, an account of the development of the method will be found in my previous article in the MEDICAL CHRONICLE (October, 1896).

It may be well now to enumerate briefly the methods which can be used for the diagnosis of the typhoid bacillus by means of the serum of immunised animals, and which were known when my investigations were begun :—

(1) *Pfeiffer's Method*, as described in the paper by Pfeiffer and Kolle (*Zeitschrift für Hygiene und Infectionskrankheiten*, February 21, 1896). A loopful (2 mgs.) of a 20 hours' culture on agar of the bacillus investigated is mixed with 0·1 to 0·2 ccm. of the serum of an animal strongly immunised against typhoid fever (or of the serum of a typhoid convalescent), this serum having been added to bouillon so as to obtain a total amount of fluid equal to 1 ccm.

This quantity of fluid containing the bacillus to be tested and the immunised serum is now injected into the peritoneal cavity of a guinea-pig weighing 300 grammes. Small drops of peritoneal fluid are then removed 15 minutes, 30 minutes, and 1 hour after the injection.

If the bacillus injected is a genuine typhoid bacillus it is at first immobilised, and after a time (if the serum be potent) it undergoes a granular degeneration, and disappears entirely, like a piece of sugar dissolved in water.

(2) *Metchnikoff and Bordet's Method*, as described in Bordet's paper (*Annales de l'Institut Pasteur*, 1895, p. 462. See specially pp. 491, 496). A young culture (20 hours) of the vibrio under investigation is mixed with 5 or 7 ccm. of bouillon, or 0·60 per cent solution of chloride of sodium. To 2 drops of this emulsion, 1 drop of the corresponding preventive serum is added. This is placed on a cover glass, so that

the drop may be used as a hanging drop, for continuous observation in the usual way. To this mixture a small quantity of the fresh serum of an ordinary animal is added when a complete reaction is wanted. The incomplete reaction can be observed without addition of fresh serum, and in this case also a very minute dose of preventive serum is sufficient. The incomplete reaction consists of immobilisation and of the formation of heaps or clumps, produced by the running together of the paralysed bacilli. After the addition of the fresh normal serum, the complete reaction is manifested by the transformation of the vibrios into granules.

Bordet considers that this reaction is based on the same principle as that of Pfeiffer, and says it is quite as specific.

(3) *Max Gruber and Durham's Method.* Though the paper by Dr. Durham, in the *Journal of Pathology and Bacteriology* (July, 1896, p. 13), was not available at the beginning of this investigation, the following account of the method which these authors have specially elaborated is taken from that communication. The bacillus to be tested is grown at 37°, for 12 to 24 hours, on agar. A loopful of the culture, equal to about 3 mgs., is made into an emulsion by thorough mixing with 0·5 ccm. of bouillon. The preventive serum, which is used as a test, is diluted with bouillon also. Generally 5 to 10 mgs. of serum are mixed with bouillon, so as to make the total amount of fluid equal to 0·5 ccm. The 0·5 ccm. of emulsion are added to the 0·5 ccm. of diluted serum, the mixture being placed in narrow specimen tubes, measuring three-eighths of an inch in diameter. In such a mixture, when 10 mgs. of serum are used, the quantity of serum is $\frac{1}{100}$ part of the whole fluid. The two fluids are well mixed, and then allowed to stand for 30 to 60 minutes. At the end of this time, if the bacillus examined be the specific one corresponding to the preventive serum used, a sediment will be found at the bottom of the specimen tube; the fluid above this will be clear. The precipitation is due to the formation of clumps, as has been described above in connection with the microscopical method of Bordet.

Durham has also used the hanging-drop method, introduced by Metchnikoff and Bordet. Durham points out, in connection with the application of this method, that complete action may be obtained between microbes of apparently different species, and that it is not absolutely specific.

It will be noticed that all the methods so far mentioned had been proposed only for the purpose of testing doubtful bacilli.

(4) *Widal's Method.*—Only one method—the slow one—had been published on the 1st of July, when I began working at this subject, but by the 29th of July a complete account of all the methods used by

Widal had been put in print (see *Semaine Médicale*, July 1, 1895, p. 259, and July 29, p. 295). I will give the account of these methods as taken from the latter communication.

A Rapid Method.—To 10 drops of a neutral bouillon culture of the Eberth's bacillus (24 or 48 hours old) is added 1 drop of blood serum separated from blood obtained from the finger of the patient. These are thoroughly mixed in the well sterilised mixing cell of the hematimetre. A drop of the mixture is taken and placed on a slide, covered, and examined at once under the microscope. A drop of the culture to which serum has not been added is used as a control. If the bacilli immediately form clumps, taking on the appearance of so many islands in an archipelago, a positive diagnosis can be made at once. If the bacilli remain mobile, a typical reaction may not be obtained till after a quarter or half hour have elapsed. If the clumps, though characteristic, are not compact enough, it may be well to examine the preparation after a few hours.

A Slow Method.—This is the method which was used by Widal in his first observations, and which he gave in his first communication. Some blood is obtained from a vein. One part of the serum under examination is mixed with 10 or 15 parts of bouillon. This mixture is inoculated with the typhoid bacillus. It is then incubated at a temperature of 37° C.; 24 hours after, in the case of a positive reaction, the bouillon is clear, and a precipitate has formed at the bottom of the tube. This precipitate is composed of well-formed clumps. This, as will be seen, differs from Charrin and Rogers' experiments with the bacillus pyocyaneus (1889) in that the serum is diluted. When the serum does not come from a typhoid patient the fluid at the end of 24 hours is uniformly turbid. Widal has also used a method similar to that described by Gruber and Durham, and which therefore does not require to be mentioned again, the two referred to here are those which he has chiefly employed.

(5) *Grünbaum's Method.*—Dr. Grünbaum's method,used by him early in the year, was published only on the 19th of September, 1896. He collects the blood in a special U-shaped capillary tube. The blood is centrifugalised. The separated blood serum is blown out on a slide.

By means of a special graduated pipette the blood serum is diluted 16 times with bouillon and thoroughly mixed with it. An emulsion of the typhoid bacillus is made by mixing a platinum loopful of a 20 hours old culture of the typhoid bacillus on rather dry agar, and 1 ccm. of bouillon. A small drop of the emulsion is placed on a cover glass. Another drop of the diluted serum, as nearly as possible of the same size as the other drop, is mixed with it. This mixture is made into a hanging drop preparation, which is examined with an immersion lens. If the reaction (immobilisation and clumping) is observed within 30

minutes the case is one of typhoid fever; it is, according to Grünbaum, impossible to say whether a negative reaction indicates a negative diagnosis.

A consideration of all these methods will show that they can be reduced to three types, if we exclude Pfeiffer's method, which is inapplicable in ordinary practice, and which has been mentioned here chiefly on account of its scientific importance:—(1) The bacillus is cultivated in a medium containing a greater or less proportion of the immunised serum (this is really a modification of Charrin and Roger's method, used as far back as 1889), the reaction being indicated by a precipitate which is visible to the naked eye after 24 hours; (2) an emulsion of the bacillus is mixed with the more or less diluted serum and allowed to stand in specimen tubes, the reaction being indicated by the formation of a precipitate which is visible to the naked eye within one hour (this is Gruber and Durham's method); (3) an emulsion of the bacillus is mixed with the more or less diluted serum, and is examined under the microscope (this is Metchnikoff's method, modified by Bordet and adopted by Widal) the reaction being indicated by immobilisation and formation of clumps visible under the microscope within a few minutes.

In my first attempts I tried these three types of methods with several variations, using in my experiments: (1) Various cultures of typhoid bacilli; (2) various dilutions of the serum of a strongly immunised animal; (3) the blood of some typhoid patients, and of other individuals. I used either the whole blood or the serum of the same blood separated by coagulation or centrifugalisation. The blood was also used after drying it for one or several days.

(*a*) The first conclusion I came to was that *a moderately attenuated culture of the typhoid bacillus*, which I had obtained from the spleen of a typhoid patient two years previously, gave invariably good results.

Cultures of this bacillus were typical in every way. They gave clearly and constantly all the reactions which have been given above as typical of the Eberth's bacillus. Their pathogenic action, which was strong soon after isolation, had become weak. A bacillus, recently isolated from a typhoid spleen and *of great virulence* to guinea-pigs, is, however, preferable to an old attenuated culture, as I have found by repeated trials. The form of cultivation which has given me the best results is that in *neutral* peptone bouillon. Cultures on alkaline bouillon or agar were unsuitable. The culture should not be older than 20 to 24 hours. It is well to know, however, that when cultivation is continued for two or three months in bouillon, re-inoculation being made every day or every other day, the cultures become so attenuated that they begin to clump of their own accord, and that the addition of any blood may cause a reaction similar to that produced by typhoid blood. Even

when this takes place there is a marked difference between the amount of clumping produced by addition of typhoid blood and of ordinary blood, but it is not safe to trust to such quantitative estimations to overcome this difficulty. The best way is to keep inoculating from time to time solid media, such as solidified serum, potato, nutrient agar or gelatine, and to inoculate the bouillon daily from such cultures, made at intervals of fifteen days, or one month. I have found that old cultures on agar regained their full activity after several months.* I much prefer, eighteen to twenty-four hours old, cultures in bouillon to emulsions made with agar or gelatine cultures of the same age, because the bacilli are more evenly distributed in the bouillon, they move more actively, and the formation of accidental clumps is much more rare.

(*b*) I was obliged to give up the test tube method recommended by Durham for three reasons. In the first place the reaction was often incomplete or ambiguous after one hour, there being either imperfect precipitation of clumps in the case of weak sera, or, in the case of non-typhoid sera, formation of small accidental clumps which could not be distinguished with the naked eye from those produced by typhoid serum.

This absence of sharpness is very marked when it is contrasted with the extraordinary clearness of the microscopical method.

Secondly, the whole blood was not so suitable as quite clear serum to produce a good reaction; this made it necessary to separate the corpuscles.

Thirdly, to get the quantity of clear serum necessary ($\frac{1}{10}$th ccm.), it was necessary to obtain a larger quantity of blood than was needed for the microscopical method.

(*c*) *The growth of the bacillus in bouillon to which typhoid or other serum had been added* was at times very typical and the clumping good, but at other times this was not the case. Clumps undoubtedly formed, but sometimes the whole fluid remained turbid. From time to time the bacillus, when grown in bouillon to which no serum had been added, produced clumps which it would be difficult to distinguish from those resulting from the action of the typhoid bacillus. This tendency to spontaneous clumping is greatly influenced by the degree of alkalinity of the media. Dr. Sidebotham, who is investigating this point, has obtained important results, which he will publish shortly.

(*d*) *The microscopical examination of mixtures of emulsions of the bacillus with serum* gave by far the most satisfactory results. The results were obtained with very small quantities of material. The reaction was very rapid, very sharp, and seldom ambiguous.

* Some of my old cultures were, however, found to be less sensitive than others, for reasons which I have not yet discovered.

After the first trials I discarded the hanging-drop arrangement, which presented no advantage over the film produced between a flat slide and cover glass. In fact, I believe that the hanging drop presents several disadvantages, which will be evident when I explain the advantages of the thin film. (An ordinary film can be prevented from drying, and can be observed from day to day for over a week by placing the preparation in a moist chamber well saturated with water vapour.)

It became also evident to me that no advantage is derived from the complete separation of the red blood corpuscles from the serum. In fact, the presence of a few red blood corpuscles is very desirable in the special method which I recommend, for the blood corpuscles act as supports for the cover glass, keeping it at a constant distance from the slide, and regulating in this way the thickness of the film. The distribution of the corpuscles, also, indicates whether the bacilli have been thoroughly and evenly mixed with the serum. Dry blood is not suitable; with it the reaction can easily be obtained, notwithstanding a certain loss of potency, but homogeneous mixtures and dosage are difficult.

In my first experiments I measured the blood serum, the bouillon, and the cultures by means of graduated pipettes, and mixed them in small capsules. When a series of consecutive observations had to be made this implied much waste of time, owing to the absolute necessity of thoroughly cleansing the pipettes which had been used for measuring the serum. This cleansing of the pipettes is a matter of no small importance, for, with strong sera the slightest trace of the active serum left about the pipette is often enough to communicate to another, and possibly inactive, serum the power to cause agglomeration. Heating of a thick pipette to destroy the activity of the serum necessitates cooling and a waste of time.

Weighing the serum, though accurate when quantities are sufficient, becomes impracticable and exceedingly slow when very small quantities of serum have to be used, and though I constantly use weighing instead of graduated measures in bacteriological work, as in water analysis for instance, I cannot recommend the gravimetric method in this case.

Having for object to find a method of measuring which would not involve much waste of time, or the use of several pieces of apparatus which would increase the chances of contamination of one serum by another, I finally came to the conclusion that the essentially bacteriological method of measuring by loops would be the best.

A small platinum loop, measuring 1 mm., internally holds about two milligrammes of fluid. This can be ascertained by direct weighing of the drops carried by such loop, and deposited on a glass slip placed in

one of the pans of a delicate chemical balance. By depositing a number of such drops with the same loop on the same glass slip, in such a way that the drops do not touch each other, one can easily ascertain that the weight of these drops is always exactly proportional to their number. If one drop weighs two milligrammes, five drops will weigh ten milligrammes, and so on.

The method of dilution I adopted was, therefore—(1) To take a certain number of drops of the bouillon culture of the typhoid bacillus and place them close together on a sterilised slide or on a sterilised cover glass; (2) to sterilise the platinum loop by heating it to redness in a flame; (3) to take one drop of the serum or blood (diluted or not with bouillon) and place it by the side of the drops of culture; (4) with the same loop to rub thoroughly all the drops together (serum may be previously diluted with bouillon exactly in the same way); and (5) to cover the mixed drops with a cover glass (if the mixture had been made on the slide), or to turn the cover glass moist surface downwards and lay it on a slide (when the mixture has been made on the cover glass).

To avoid any error it is well in all cases to make a similar preparation with or without the addition of normal serum, to serve as a control showing the exact state of the cultures as regards mobility of the bacilli and tendency to clumping.

Under these conditions, *agglomeration, if it is likely to appear, is usually clear within one to five minutes*, and is clearly indicated within the first half-hour. But it sometimes becomes more distinct at the end of one or two hours. On the other hand, if agglomeration does not become evident within the first two hours it is not likely to occur except in a very imperfect fashion afterwards. I have generally seen blood of non-typhoid persons, either normal or diseased, remain for days in presence of the motile bacilli, under these conditions, without any agglomeration being produced. I have never seen a normal blood or the blood of febricants give within two hours any definite agglomeration, and it is very rare for such blood, even in the proportion of one to five or one to two, to give any reaction after twenty-four hours.

Dr. Sidebotham, also, who has examined a large number of cases by this method, has obtained similar results. In presence of these results, I have been much surprised to hear of the very unsatisfactory results obtained by Dr. Durham (*Lancet*, Dec. 19, 1896), who informs us that out of ten typical cases of typhoid fever only six reacted and four gave no reaction. This can only be explained on the supposition that the method he uses does not give a clear reaction.

The results obtained by Grünbaum (*Lancet*, September 29 and December 19, 1896) are more in accordance with mine, but still I

cannot account for the fact that he should have found it so easy to get agglomeration of bacilli with the blood of patients not affected with typhoid fever.

It is impossible to avoid the conclusion that the methods recommended by these authors, though useful for the purpose of working out certain questions, are unsuitable for current diagnostic work ; and without disregarding the excellent work done by them I prefer to use the method I have adopted.

It will be noticed that by this method the chances of contamination are reduced to a minimum. It is also worth noticing that the bacilli are lodged in thin films of constant thickness, occupying a capillary space, and that they cannot run together to form clumps without their being compelled to do so by some power overcoming resistances caused by these physical conditions.

In concluding this paper I wish to acknowledge gratefully the great assistance I have received from Dr. Niven and Dr. Marsden whenever I required material for investigation or information about cases.

[*Sero-diagnosis of Glanders.*—The limits of this paper prevent me alluding here in full to other observations which I have made in connection with diphtheria, tuberculosis, and glanders. Up to the present time I have not obtained very satisfactory reaction with the tubercle bacillus, but this is probably due to mechanical difficulties which I hope to overcome. In the case of glanders or farcy I have obtained results which are almost more striking than those obtained in the case of typhoid fever. To obtain good results it is necessary to use suitable cultures. Those which have given me the best results have been three days old cultures on slightly acid potato (cultures in alkaline bouillon are unsuitable); an emulsion is made of two or three loopfuls of such a culture with two or three drops of bouillon. Nine loopfuls of the emulsion are mixed with one loopful of blood serum from a horse affected with glanders, and the specimen examined at once. The clumping in some cases appears at once. Though I have tried for several months to obtain blood from a horse affected with glanders or farcy, it is only lately that I have been able, through Mr. King's kindness, to apply the test. My friend Professor MacFadyean has already published a short note, in which he announces the occurrence of this reaction, in the *Journal of Comparative Pathology.*]

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