THE THERMOPRECIPITIN METHOD IN THE DIAGNOSIS OF BUBONIC PLAGUE IN CADAVERS.

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The diagnosis of bubonic plague in rat cadavers, the examination of which is a recognised method of prophylaxis for ships or other communities, which may have been in contact with endemic centres, is attended by especial difficulties, and, on the other hand, calls for especial speed and finality in technique.

It rests at present finally on the isolation and identification by the serum reaction (agglutination) of the *B. pestis*. Whether the organism is cultivated directly from the cadaver or indirectly by interposition of an experimental animal, this requires a delay of at least two days, in a matter in which time is of the highest importance, as, for instance, when a suspected ship is detained pending a decision. But for agglutination a pure culture is necessary, and to obtain this is a matter of difficulty owing to the speed with which *B. pestis* is overgrown at its optimum growth temperatures by bacteria of intestinal and saprophytic origin. The difficulty becomes an impossibility when, as often happens, the cadavers arrive at the laboratory in a state of decomposition, especially in hot climates, *B. pestis* having very little resistance to high temperatures (Dunbar and Kister, Kister and Schumacher).

On these considerations any diagnostic aid which would obviate the necessity of isolation and pure cultivation of *B. pestis*, would be welcome. Such a diagnostic method is the "Thermoprecipitin Method" devised by Ascoli for the detection of anthrax in like circumstances.

This research is an attempt to discover how far it may be of use in the especial case of bubonic plague. The experiments were already

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well advanced, when the work of Piras, on the same subject, appeared in the *Centralblatt für Bakteriologie*, Sept. 1913, and were thereafter continued on the same lines on which they had been begun. Piras' results are discussed below.

Ascoli's method consists in bringing together anthrax serum of high value and decoctions of the suspected tissues, the occurrence of precipitation being taken as proof of the presence of anthrax. The decoctions are prepared by emulsifying the tissues in normal salt solution, heating for some minutes in the water-bath at boiling temperature, and filtering. This process decolorises and eliminates coagulable proteid from the extract, without influencing adversely the final reaction, since the bacterial antigens concerned are known to have a very high resistance against heat (Nicolle, Pick). Ascoli proved that:

(1) Extracts of anthrax-affected organs gave a precipitate similar to that given by extracts of *B. anthracis*, when brought in contact with anthrax-immune serum of high value.

(2) This precipitate was specific;

(3) and was still demonstrable in organs in which, owing to more or less advanced putrefaction, the usual bacteriological methods were unavailable.

(4) The reaction might give a negative result, though the case had actually been one of anthrax, when the number of bacilli in the organs at death had been inconsiderable—a conclusion to be expected on *a priori* grounds.

Technique. The technique followed the lines laid down by Ascoli. The organs (heart, spleen, liver, and—where found—diseased gland, as being those in which the bacteria occur in greatest number), were, after appropriate bacteriological examination, by means of film preparations, cultures, and inoculation of experimental animal, removed from the cadaver, minced with the scissors, and then gently shaken up with sterile normal salt solution in the proportion of 4–5 c.c. solution to 1 gramme of organ. The flask containing the suspension was plunged into boiling water for five minutes, the suspension allowed to cool, filtered through doubled filter-paper, if necessary, several times, till quite clear or at most slightly opalescent, and the filtrate used for the precipitation experiment. This was carried out by the "ring" or "Schichtungsmethode" of Ascoli, viz. a few drops (c. 0·25 c.c.) were transferred to a small glass tube, and a corresponding volume of serum introduced by means of a glass pipette drawn out into a capillary tube, the capillary end being brought to the bottom of the tube, before the

serum was allowed to flow out. The two fluids thus remained in two distinct layers, the heavier serum below. The presence of specific antigen in the extract, *i.e.* a positive reaction, was indicated by the appearance of a whitish ring at the surface of contact. The glass tubes were 7 cms. deep by 4·5 mm. diameter, and were made, according to the experience of Meyer, with flat instead of rounded bottoms, in order to eliminate the optical disturbance caused by refraction from a curved surface. They could easily be cleaned under a small jet of cold water, and so used again. The tubes were mounted on a black stand to the back of which an adjustable black screen was affixed. The rats used were the ordinary white laboratory species. They were infected by subcutaneous injection of $\frac{1}{5}$ to $\frac{1}{10}$ platinum loop of a 24 to 48 hour agar slope culture, and succumbed on the 3rd to 5th day. The strains of *B. pestis* were two which had been cultivated in the Institute for more than ten years, and were those used in the production of the " Berner " polyvalent pest serum. One strain originated from St Petersburg, the other from the laboratory of Prof. Schottelius in Freiburg-in-Breisgau. " Berner " pest serum, prepared in the Institute according to Yersin's method, or a mixture of " Berner " and Cronstadt sera, was employed. Details of technique, which differed in different parts of the work, are given with the corresponding table.

The specificity of the reaction. Kraus found that plague-immune serum caused a precipitate in extracts of *B. pestis*, but not of *B. typhi*, nor of the cholera vibrio ; Zlatogoroff that the reaction was negative also in the case of the bacteria most nearly allied in morphology and pathogenicity to the *B. pestis*, viz. *B. gallinarum* of fowl-cholera, *B. suisepticus* of swine-fever, *B. septicaemiae* (Koch, Gaffky), and *B. pseudotuberculosis rodentium* of Pfeiffer. McConkey, on the other hand, states that the last-named causes a precipitate, though quantitatively less than the specific, in plague serum.

Kolle and Otto examined 50, Zlatogoroff 22, strains of *B. pestis* from epidemics all over the world, and found that culture extracts of each gave a marked reaction with the sera used by them.

The results of the present author's experiments with these and other bacteria are incorporated in Table A. In the case of each disease the precipitation experiment was carried out with (*a*) extracts, prepared as described above, from the fresh organs, after bacteriological examination had shown the presence therein of the causal bacteria in great numbers ; and (*b*) filtrates from suspensions in normal salt solution of the bacteria.

TABLE A.

Bacterium	Bacterial Extract + Normal Serum	Bacterial Extract + Plague Serum	Organ Extract + Normal Serum	Organ Extract + Plague Serum
Normal organs			−	−
B. pestis (1)	−	+++	−	+++
„ (2)	−	+++	−	+
B. coli murium (isolated from normal rat faeces)	−	+		
Saprophyte isolated from cadavers	−	+		
B. gallinarum:				
(1) from Kralsche Museum, Vienna	−	±		
(2) from L. W. Gans, Frankfort.. ..	−	±		
B. suisepticus:				
(1) from Kral. Mus., Vienna	−	±	−	±
(2) from L. W. Gans, Frankfort.. ..	−	±	−	±
B. dysenteriae	−	±	−	-
B. typh.	−	+	−	−
Diplococcus pneumoniae	−	±	−	−
B. paratyphosus B ..	−	±	−	−
B. paratyph. Dansyz ..	−	−	−	−
B. pseudotuberculosis rodentium of Pfeiffer	−	±	−	−

The bacterial filtrates were prepared as follows: A 48-hour agar slope culture was emulsified in 6 c.c. of normal salt solution, mechanically shaken for two hours, plunged into boiling water for five minutes, and finally filtered through a porcelain column. In the case of *B. pestis*, Kraus' experience that filtrates from old (14–30 days) bouillon cultures give an intense reaction, was confirmed, but bouillon was rejected as an extractive medium, because the specific gravity of bouillon plus bacterial extractives may be so near that of serum that a sharp surface of contact is difficult to obtain.

With each experiment a control was made with normal serum and the filtrate concerned. The plague serum was tested from time to time with a plague bacterial extract of known value.

The experiments were carried out at room temperature (18°–22° C.), and the results kept under observation for 20 minutes, and then noted again after having stood overnight, *i.e.* for about 16 hours. The degrees of the positive reaction are indicated in the table as follows:

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+ + + = flocculent ring within 15 minutes, deposit in 16 hours,
+ + = well-marked ring without distinct flocculi, in 15 minutes, deposit in 16 hours.
+ = cloud in 15 minutes, distinct deposit in 16 hours.
± = cloud in 15 minutes, no deposit in 16 hours.
− = no cloud, no deposit.

In the case of *B. pestis* extracts, the reaction always appeared immediately as a bluish-white ring at the surface of contact, waxed steadily in intensity during the first 15 to 20 minutes, during which the experiment was under constant observation, and, after 16 hours, showed itself as a flocculent deposit at the bottom of the tube, the disturbed surface of contact above showing a secondary diffuse flocculent precipitation-ring. No reaction was observed with normal serum and plague extracts within 20 minutes, though a faint turbidity was noticed once or twice after 16 hours. Normal salt solution, *i.e.* the medium used for extraction, in contact with plague-immune serum, was likewise consistently negative.

The unspecific reactions may be eliminated by dilution of either extract or serum. Two different *B. pestis* extracts still reacted with serum at a dilution of $\frac{1}{200}$, whereas the extracts of *B. coli*, *B. gallinarum*, and a saprophyte isolated from a cadaver, failed to react with serum at a dilution of $\frac{1}{2}$, the positive reaction being taken as the appearance of a ring or cloud at the surface of contact within 15 minutes.

The reaction in decomposing cadavers. The experiments are in three series, in each of which the treatment of the cadaver after death differed.

Series I. The cadavers were put unopened into glass jars open to the atmosphere through a wire-gauze lid, were covered with moist earth, and were kept in a cellar, at a temperature which varied between 10° and 18° C. At intervals a cadaver was removed and opened under precautions against introduction of foreign organisms. A guinea-pig was infected with material from liver and spleen. The method of infection of the guinea-pig is indicated in each case in the table.

The results are incorporated in Table B.

From these seven preliminary experiments appear the facts (1) that, although the cadaver, in which decomposition was most advanced, and which yet contained living virulent *B. pestis*, gave no precipitation ring, a 29 days' old cadaver reacted strongly positive, while two 10 days' old were negative; and (2) that all three positive precipitation results coincided with positive inoculation experiments. A possible explanation of both observations lay in the bacterial content of the

TABLE B.

Experiment	Days in earth	Degree of decomposition	Smear preparations	Method of inoculation of guinea-pig	Result of inoculation	Cause of death	Precipitation experiment
1	8	Slight	Numerous saprophytes ? Plague B +	Subcutaneous injection of bouillon emulsion spleen and liver	✠ 5th day	Plague +	+ +
2	8	„	Numerous saprophytes Plague B +	„ „	✠ 6th day	Plague +	+ +
3	10	„	? Plague B + ?	„ „	Still alive and healthy 26th day	Plague −	−
4	10	„	−	Inunction of shaved abdominal skin with spleen and liver	Still alive and healthy 26th day	Plague −	−
5	32	Moderate	−	Pea-sized piece of liver and spleen in abdominal skin pouch	✠ 7th day	Plague +	+ +
6	39	„	−	„ „	Alive and healthy 21st day	Plague −	−
7	68	Advanced	−	„ „	✠ 4th day	Plague +	−

organs at death. Where that had been considerable, precipitation and inoculation would both tend to a positive; where inconsiderable, to a negative result.

Series II. Accordingly, in the next series a rough estimate was made of the comparative number of plague bacilli in the heart-blood of each cadaver at death. As soon after death as possible, the heart was exposed by a small incision through the chest wall, opened, and several films made from the blood. The bacilli in a number of microscopic fields of each film were counted, and the average reckoned. The results are entered thus: *Few* = less than 3, *considerable* = between 3 and 10, *numerous* = more than 10 bacilli in one field of the oil immersion lens. As will be seen from Table D, which shows the comparative bacterial contents of blood, bubo, spleen and liver in 12 fresh cadavers, a correspondence does exist between the number of bacteria in the circulation and that in the spleen and liver. This examination of the fresh cadaver necessitated its being dipped into absolute alcohol for a few minutes before being opened, in order to kill any living fleas which might be in the fur. The alcohol was afterwards allowed to completely evaporate before the cadavers were stored away. In this series they were kept as before in glass jars with wire-gauze lids, but they were covered with dry grain, and the temperature of the storage room was 28°–30° C.

After the first two experiments the remaining cadavers were allowed to freeze in the open at a temperature of – 6° C. and then returned to the incubator. The object was to kill off the plague bacteria, the resistance of which to variations of temperature is known to be low, though that to constant low temperatures is high. In all the smear preparations made direct from the organs, numerous saprophytes were seen, but fewer than in the films from Series I. The cultural examination was carried out on the lines of the practice in the Hamburg Hygienic Institute: from each organ a bouillon and an agar-plate medium were inoculated, the former by introduction of a small piece of organ, the latter by gently drawing across it the fresh cut surface of the organ. The bouillon was incubated at 30° C. and examined after 24 hours. If it contained polar-staining Gram-negative organisms, a second series of agar plates was inoculated from it. Both series of agar plates were kept in the ice chest at + 6° C., and showed the first colonies in five to seven days, *i.e.* in the same time as *B. pestis* under the same conditions. All the cultures were negative, the agglutination test with plague serum being applied to all doubtful organisms.

All the experimental inoculations were carried out by the abdominal skin-pouch method.

In the description of the degree of decomposition of the cadaver, "*moderate*" = strong smell of decomposition, organs easy to recognise; "*advanced*" = organs recognised with difficulty. Bubo, though searched

TABLE C.

Experiment	Bacteria in blood at death	No. of days between ✠ and experiment	Degree of decomposition	Smear preparations	Cultures	Result of inoculation of guinea-pig	Result of precipitation experiment
1	Few	8	Slight	–	–	+ ✠ on 2nd day	+
2	Numerous	15	Moderate	–	–	–	+ +
3	Few	21	,,	–	–	–	–
4	Numerous	21	,,	–	–	–	+ +
5	Numerous	25	Advanced	–	–	–	+ + +
6	None	25	,,	–	–	–	–
7	Few	28	Moderate	–	–	–	–
8	None	28	,,	–	–	–	–
9	Considerable	31	,,	–	–	–	+
10	Few	31	,,	–	–	–	±
11	Few	39	,,	–	–	–	+
12	None	39	,,	–	–	–	–
13	Numerous	43	,,	–	–	–	+ +
14	Considerable	43	,,	–	–	–	+
15	Few	45	Advanced	–	–	–	+
16	Considerable	45	,,	–	–	–	+ +
17	Few	49	,,	–	–	–	+
18	Numerous	49	,,	–	–	–	+ +
19	Considerable	49	,,	–	–	–	+

for, was never recognised, although the rats had received the same subcutaneous dose as those in Table D, where bubo was found in 11 out of 12 cases. This is in accordance with Zlatogoroff's experience that the bubo is the first organ to disappear, as the result of decomposition, the spleen being the next to follow.

Of the 19 cases, 13—*i.e.* well over half—gave a certain positive precipitation, one a doubtful, and five a completely negative result. Between the result and the degree of putrefaction of the cadaver no sort of correspondence appears, whereas that between the result and the bacterial content of the blood seems as exact as the method of determination of the latter can permit. Thus, the five negative cases occur where the bacteria were "*few*" or none, the six most strongly positive where the latter were "*considerable*" or "*numerous*," and in none of the four cases where "*few*" bacteria correspond to a positive result, does that result reach more than a + degree.

Series III. The procedure after death was the same as in II, but the cadavers had been left so long in the incubator that all were completely mummified, hard, and parchment-like. No organs could be distinguished, therefore to obtain tissue for the experiment the dried-up contents of the upper half of the abdomen and of the chest were scraped out. Bacteriological examination was omitted. In 9 of the 14 cases the bacteria in the blood had been *few* or none, in one "*considerable*," in four "*numerous*." In one of the latter, which had been stored for eighty-five days, a positive reaction + + was obtained, the remaining cases being negative. The ages of the cadavers ranged from 71 to 100 days, from which must be concluded that putrefaction has not a great, yet a certain influence on the bacterial antigen. In actual experience such mummified cadavers are sometimes sent for examination, but the question has little practical value, since a number of cadavers are sent at the same time, in which case one more suitable may be chosen for experiment (Dunbar and Kister, *l.c.*). In this series the first experience was made of a difficulty mentioned by Ascoli—that of obtaining a clear filtrate from the putrefied tissue. Even after repeated filtration or centrifugalisation, several of the extracts remained opalescent. A slight opalescence did not however obscure a positive reaction, but repeated filtration was a disadvantage when small volumes of fluid were being dealt with, since much is lost by evaporation. Centrifugalisation or filtration through asbestos in a small funnel such as that sold by Messrs L. W. Gans, Frankfort, with the "Ascoli Anthrax Diagnostikum," is to be preferred. With such an opalescent solution

a control with extract alone should be carried out in case the extract alone gives a sediment in 16 hours. A clear solution is more readily obtained when the extract is allowed to cool almost completely before filtration.

The reaction in fresh cadavers (up to 48 hours old). Rats 1 to 6 inclusive were injected with $\frac{1}{10}$, 7 to 12 with $\frac{1}{5}$ platinum loop of an agar slope culture. Post-mortem appearances were typical of acute plague, bubo being recognised in all but one case.

Extracts were made from the blood, bubo, spleen, and liver in each case, each organ being first examined as to its bacterial content. In making the films for this purpose, the fresh section of the organ itself was drawn over the coverslip. The results are collected in Table D. The extracts referred to as "*heart-blood*" were made from heart and heart-blood together, the blood alone having been examined in films. The extracts from bubo were invariably opalescent, those from heart-blood occasionally so. Piras states that he obtained clear filtrates by using distilled water instead of normal salt solution as extracting medium. Distilled water was accordingly tried in experiments 4 and 5, the resulting filtrate from the bubo being as before opalescent, and the precipitation result as mentioned below. No bubo was found in experiment 11.

TABLE D.

	Heart-Blood		Bubo		Spleen		Liver	
Experiment	Bacterial Content	Precipitation	Bacterial Content	Precipitation	Bacterial Content	Precipitation	Bacterial Content	Precipitation
1	Few	–	Numerous	+ +	Few	–	Few	–
2	None	–	,,	+ +	Few	–	Few	–
3	None	–	,,	+ +	Few	–	Few	–
4	Considerable	v. below	,,	v. below	Numerous	v. below	Numerous	v. below
5	Few		,,		Few		Considerable	
6	None	–	,,	–	Few	+	Few	–
7	Few	–	,,	+ +	Considerable	+	Considerable	+
8	Considerable	–	,,	+ +	Numerous	+ +	Numerous	+ +
9	None	–	,,	+ +	Few	–	Few	–
10	Few	–	,,	+ + +	Few	–	Few	–
11	Considerable	–			Numerous	+ +	Numerous	+ +
12	Few	–	Numerous	+ +	Considerable	+	Considerable	±

The bubo gave a marked positive result in every case but one, where the negative result must be due to a technical failure, since the bacteria were "*numerous*." The spleen reacted positive in six, the liver in three, of the 10 cases. From an examination of the relative bacterial content of the organs other than bubo, it will be seen that a bacterial content of the spleen and liver large enough to give a positive precipitation result, is only present when septicaemia of more or less degree had been

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present at death, a condition by no means always to hand. In Piras' communication no mention is made of this fact, his tables showing constant positive results, although, as seen in the preceding Tables B, C, and D, the degree of septicaemia, even in rats of like size infected with the same quantity of culture, shows all possible variations between the two extremes. Piras further states that he prepared the extracts with distilled water. Now, not only did all the organ extracts from experiments 4 and 5, Table D, which were made with distilled water, react strongly positive, regardless of their bacterial content, but also distilled water itself gave a marked positive result with plague serum, but no trace with normal. This unspecific "*ring*," however, disappeared, leaving no deposit in 24 hours, the period which Piras allowed to elapse before entering his results.

Conclusions. (1) The "Thermoprecipitation" reaction between plague-immune serum and extracts of plague bacilli, whether obtained from pure cultures or from infected organs, is specific;

(2) and can therefore be used to diagnose plague in cadavers, under the following conditions: the occurrence of a marked positive reaction (+++, ++, or +) between undiluted serum and an organ extract is, if the experiment is carried out with suitable controls, viz.

(*a*) plague serum + extractive medium (normal salt solution) = negative,

(*b*) plague serum + *B. pestis* culture extract = positive,

(*c*) normal serum + extract concerned = negative,

(*d*) extract, if opalescent, alone = no deposit,

in itself absolute proof that the organ was infected with *B. pestis*. A doubtful reaction (±) is merely suspicious of plague, while a negative is of no value, since the organ, though from a plague cadaver, may have contained an inconsiderable number of bacilli.

(3) Because of this last fact, the precipitation can never replace the usual bacteriological methods;

(4) but by reason of its speed, simplicity, independence of climatic conditions, or putrefaction in the cadaver, and availability in addition to the usual bacteriological methods, it is a valuable supplement to them.

(5) The further application of the method rests on the facts already known as to the distribution of the bacilli in the different organs in the various forms of the disease. Where acute bubo is found—and it is a characteristic appearance also in pulmonary plague and in that contracted by gnawing infected cadavers—the extract would give a positive

result, a negative being definitely against a diagnosis of plague. After the acute bubo, the spleen, and next the liver are the most likely organs to give a positive result. In chronic disease, where, even in the encapsulated local focus, the bacilli are usually scanty, and death takes place from toxaemia, the method would leave the bacteriologist in doubt.

These observations apply particularly to the disease as transmitted by flea-bites, of which infection by subcutaneous injection, as in this research, is the artificial imitation. In the plague cases mentioned by Dunbar and Kister as examined at Hamburg between 1901 and 1903, 59 positive cadavers were sent from four ships. In the case of cadavers from two ships, bubo is expressly mentioned, in all, the macroscopic appearances are described as "typical," suspicious bacilli as "numerous" in the organs. The precipitation method would probably have settled the diagnosis in the four cases within an hour.

In any case, the method recommends itself for trial under actual conditions in a laboratory where the examination of rat cadavers for plague is part of the routine.

NOTE.—The fowl-cholera and swine-fever cultures and sera used in the experiments were gratuitously supplied by the firm of L. W. Gans, Frankfort-on-Main, to whom acknowledgment is made.

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