

STUDY OF TUBERCLE BACILLI ISOLATED FROM CASES OF SURGICAL TUBERCULOSIS (CHIEFLY BONE AND JOINT) IN THE SHEFFIELD AREA.

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HISTORICAL.

GENERAL interest was first aroused regarding the respective powers of the human and bovine types of the tubercle bacillus to infect the human subject by the address of Koch at the Tuberculosis Congress in 1901. In this he stated that the two types produced different diseases, and that the amount of human tuberculosis due to infection by the bovine type of the tubercle bacillus was negligible.

Following this much work was done with contradictory results until the investigations of the British Royal Commission on Tuberculosis (1904–1911) put the whole matter on a more or less sound basis. The work of this Commission gave, in addition, a fresh impetus to this study and the various forms of tuberculosis received more minute investigation. As we are chiefly concerned here with bone and joint tuberculosis we shall consider this form to the exclusion of all others.

Fraser in 1912 issued a report of a study of the types of bacilli present in 70 cases of bone and joint tuberculosis in children. This showed an alarmingly high percentage of "bovine" type of infection, and drew attention to the dangers of a milk supply infected with the tubercle bacillus.

Further work, though on a larger scale, was carried out by Eastwood and F. Griffith (1916), and also by A. S. Griffith (1916), and, though not confirming the high percentage of "bovine" infections as shown by Fraser, they demonstrated that the amount of such infection was considerable. The following investigation regarding the types of the infecting bacilli in cases of surgical tuberculosis was carried out at the request of Dr Pattison, Superintendent of the King Edward VIIth Hospital for Crippled Children, Sheffield, and we are indebted to him for all the specimens of pathological material as well as for notes on the various cases.

We have also taken advantage of this work to try and demonstrate the close relationship existing between the amount of human infection due to the bovine type of the tubercle bacillus, and the amount of the milk supply infected with it.

Method of procedure. The pathological material was collected by Dr Pattison with aseptic precautions, and sent to the laboratory within 48 hrs. of its

removal from the patient. On its arrival at the laboratory it was at once examined by means of direct film preparations, and was then inoculated on to an agar plate which was incubated aerobically at 37° C. for 24 hrs. If no growth took place two guinea-pigs were inoculated subcutaneously in the region of the right groin with the pathological material, and primary cultures were attempted on various media. If, however, growth took place on the agar plate the specimen was treated with antiformin before the guinea-pigs and the culture-tubes were inoculated. In addition two other guinea-pigs were inoculated subcutaneously with the untreated material on the off-chance that the organisms, other than the tubercle bacillus, might not prove lethal. If the primary cultures proved negative further cultures were made from tuberculous material obtained from the infected guinea-pigs. This was always derived from caseous superficial or deep inguinal, or lumbar glands.

In due course subcultures were made from the primary cultures, and the growth of the various strains on divers media was studied over prolonged periods.

The subcutaneous and intravenous inoculation of rabbits with definite doses of the bacillary emulsion derived from each strain was also carried out with a view to determining the type of the infecting organism.

Description of the pathological material examined. Fifty specimens were examined—eight of these being obtained from adults, and the remaining 42 from children. In some of the children's cases more than one specimen was examined. In fact four specimens were received from one patient, and in four other cases two specimens from each were investigated.

The number of separate cases in the 0-16 years group was thus reduced to 35.

With the exception of six cases of glandular tuberculosis all the others were examples of bone and joint disease.

The following list shows the number of all the cases examined in relation to the regions from which the pathological material was obtained: Hip = 10: Spine = 8 (1 adult): Knee = 6 (1 adult): Ankle = 3 (1 adult): Rib = 3 (2 adults): Sternum = 2: Cervical glands = 5 (3 adults): Axillary glands = 1: Elbow = 2: Finger = 1: Spine and Hip combined = 2. In the examination of the 50 specimens it was found that 14 per cent. contained other organisms in addition to *B. tuberculosis*, i.e. one of the specimens contained *Streptococci*, two others *Staphylococci*, and the remaining four both *Streptococci* and *Staphylococci*.

TREATMENT OF SPECIMENS CONTAINING OTHER ORGANISMS BESIDES THE TUBERCLE BACILLUS.

Two methods were tried to prepare these specimens for inoculation:

1. The glycerine method as described by C. C. Twort (1922).
2. The antiformin method as described by Eyre (1916).

The first was found to be tedious, and not reliable for cultural work. The second was fairly reliable; but required careful technique if good results were

to be obtained. In three cases it had a lethal effect on the tubercle bacillus as well as on the other organisms present.

The difficulty is naturally found to be in the correct estimation of the strength of the mixture of antiformin and specimen, and of the time through which the antiformin should be allowed to act. The temperature too is an important factor.

We have found that the method which gives the best results is to keep the temperature and the strength of the mixture constant, and to vary the time. In other words, we use a mixture of equal parts of antiformin and specimen, and carry out the reaction at 37° C. Specimens are taken at 5 mins., 15 mins., 30 mins., and 60 mins., and separate inoculations are made on to culture media and into animals with each specimen. As routine we always inoculate two guinea-pigs with the untreated material, as, in a surprising number of cases, the animals survive the inoculation of the additional organisms.

EXAMINATION OF DIRECT FILM PREPARATIONS FOR THE PRESENCE OF *B. TUBERCULOSIS*.

By this we mean the presence of acid- and alcohol-fast bacilli in direct film preparations stained by Ziehl Neilsen's well-known method.

Various means were tried to concentrate the bacilli, *e.g.* antiformin with and without ligroin, digestion of the pus followed by centrifugalisation; but the ordinary method by which thick films were prepared and examined for an adequate period—half an hour—was found to be as efficient as any other method tried.

We found 48 per cent. of all the specimens investigated positive to this examination; but after deducting the specimens obtained from glands, and also the specimens from cases previously examined the percentage of positive findings from cases of bone and joint tuberculosis was found to be 51 per cent.

Morphology. Nothing of importance concerning the type of bacillus could be ascertained from studying its morphological characteristics. The size and granularity of the various organisms were found to depend greatly upon their environment.

Cultural characteristics. Primary cultures on various media were attempted with a view to testing the efficiency of such media for the isolation of the tubercle bacillus. The following list shows the type of medium used with the percentage of positive results obtained.

The synthetic medium was made according to the formula of Borrel, Coulon, Boez, and Quimaud (1922); the Williams' medium according to that of Williams and Burdick (1916); whilst the boiled blood agar and the peptic blood agar were made as described by McIntosh (1922) and Fildes (1920) respectively. We followed the method which is mentioned in the 2nd Interim Report of the Royal Commission on Tuberculosis (1907) in the preparation of ox and glycerinated ox sera. All the other media were made as described by Eyre (1916), except that the adjustment of the reaction, when necessary,

	Per cent.		Per cent.
Glycerine Egg	67	Broth	12
Dorset's Egg	62	Agar	10
Synthetic Medium	43	Glucose Agar	5
Haricot Broth	39	Litmus Milk	5
Glycerine Serum (Ox)	38	Gelatine	0
Williams' Medium	19	Carrot	0
Serum (Ox)	19	Beet	0
Loeffler's Serum	16	Turnip	0
Glucose Broth	16	Parsnip	0
Boiled Blood Agar	16	Glycerine Carrot	0
Peptic Blood Agar	16	" Beet	0
Potato	15	" Turnip	0
Glycerine Potato	15	" Parsnip	0
Haricot Agar	12		

was carried out as described in *Reaction of Media*, No. 35 of the Special Report Series of the Medical Research Council (1919). The carrot, beet, turnip, and parsnip media were prepared in a similar manner to that used in making the corresponding potato media.

The marked advantage of egg media was expected, but the high percentages obtained with synthetic and haricot broth media were somewhat surprising. We have found these liquid media especially useful *e.g.* for subculturing and for the inoculation of guinea-pigs. The relatively low figures obtained for serum media were no doubt due to the fact that many of the pathological specimens digested these media (50 per cent.). From those specimens which had this digestive effect growth was obtained in 8 per cent. on serum media, but in 75 per cent. on other types of media. The corresponding figures in the case of the non-digesting inocula were 66 per cent. for serum media, and also 66 per cent. for types of media other than serum. From this it would appear that if this digestive effect could be prevented serum media would be greatly improved as a primary culture medium for the tubercle bacillus. This was effected by neutralising the serum to P_H 7.0 before inspissation. Phenol-red was used as the indicator, and was incorporated in the medium. The resulting brownish pink tinge was also found to facilitate the examination for the presence of growth. In addition to preventing the digestion this medium proved to be much superior to the ordinary inspissated serum for the growth of the tubercle bacillus, as growth occurred earlier and was also more luxuriant. The following experiment further demonstrates the benefit of using this neutralised inspissated serum.

A series of old cultures of various strains of the tubercle bacillus were inoculated on to ordinary and neutralised inspissated serum. They were 23 in number; but growth was only obtained in the case of 14. (Many of these old cultures were later proved by inoculation into guinea-pigs to contain only dead bacilli.) Of these 14 strains seven grew on both types of medium, one grew on ordinary serum alone, and six grew only on the neutralised serum.

Another useful feature of the neutralised inspissated serum is that its surface is much softer than in the case of the ordinary inspissated serum, the surface of the latter being notorious for its elastic consistency and consequent difficult

inoculation. It is quite a common experience in the inoculation of, say, six tubes of the ordinary inspissated serum with the tubercle bacillus to find that growth only takes place in four or five of them. This is not such a common occurrence when one uses the neutralised preparation.

A glycerinated neutralised serum medium has also been used with success.

Classification of strains by cultural methods. The various strains were classified according as to whether they were dysgonic or eugonic. Further subdivision of these two groups by cultural tests was not found practicable. This was chiefly due to the introduction of the personal factor, as no two observers had the same idea of the amount of growth necessary to allocate a strain to its proper sub-group.

Bovine types. Nine strains were examined, and all proved to be dysgonic. Six of these were used as controls, and were obtained from cow's milk. The other three were derived from human sources.

Human types. 44 were examined, and all were eventually proved to be eugonic though only after a varying number of subcultures on different media had been studied. Three strains in particular were exceedingly difficult to group, and had to be subcultured on numerous occasions. Towards the end of this investigation we found that certain strains of the human type which appeared to be dysgonic could be made to grow much better by incubating them at room-temperature. A means, which was latterly used, was to remove the primary culture from the 37° C. incubator after growth had taken place, and subcultures had been inoculated from it, and to continue its incubation at room-temperature. This meant that the culture was incubated for about 2 months at 37° C., and for 8 to 10 months at room-temperature.

The human types of the tubercle bacillus generally grew better on glycerinated media, though this was by no means an absolute rule. Quite a number grew equally well on the corresponding non-glycerinated preparations, and in one case, which was definitely eugonic, the bacillus grew better on the latter media. For these tests egg, ordinary and neutralised serum, potato, and their corresponding glycerinated preparations were used. Three tubes of each were always inoculated in order that a better estimation of the average growth could be obtained, and each organism was carefully studied through from five to ten subcultures extending over a period of from 1 to 2 years.

As another means of differentiating the types of the tubercle bacillus Calmette and Guérin's (1908) bile potato media were given a trial. These were prepared with both human and ox bile and tested with a series of known human and bovine types of the tubercle bacillus. Ordinary and glycerinated potato were used as controls. We found these media no better for differentiating the types of the tubercle bacillus than the ordinary media at present in use for that purpose.

In conclusion we are of the opinion that, as a means of differentiating tubercle bacilli into human and bovine types, cultural methods, such as the

above-described are helpful though tedious; and that, without the results of the animal inoculation tests to guide us, they are thoroughly untrustworthy.

ANIMAL INOCULATIONS.

Guinea-pigs. In every case two guinea-pigs were inoculated with the morbid material; but as none of the strains proved atypical it was never necessary to inoculate further guinea-pigs with known doses of bacillary emulsion. One guinea-pig was always allowed to die from the result of its tubercular infection, and the other was killed as occasion demanded. Every specimen proved lethal to guinea-pigs, and produced generalised progressive tuberculosis in them.

The three bovine types killed off the respective guinea-pigs in 55, 104, and 104 days.

The 47 human types killed off the guinea-pigs in an average of 88 days (maximum = 188 days, minimum = 43 days).

On account of the small number of "bovine" infections, and also because no fixed dose was used, an accurate comparison between the effect of the inoculation of human and bovine types of the tubercle bacillus into guinea-pigs cannot here be made.

Rabbits. On examining the literature dealing with the presence of human and bovine types of the tubercle bacillus in certain groups of human tuberculosis we were struck with the remarkable difference between the results obtained by different observers, *e.g.* Fraser (1912) and A. S. Griffith (1916) examined cases of bone and joint tuberculosis in children under 12 years from the same district (Edinburgh) and found respectively that 65.6 per cent. and 26.3 per cent. were due to infection by the bovine type of the tubercle bacillus. This may be due to the use of different techniques, or to the fact that rabbits of different breeds and from different localities may react differently to the test dosages. We have tried various methods of technique, and have adopted that described by A. S. Griffith (1911). The chief point to watch in this method, and one in which grave errors may arise, is in the removing of the bacilli from the three weeks old ox serum cultures. There is a great risk of incorporating some of the medium in the bacillary emulsion especially when working with dysgonic growths. In this way serious errors in the estimation of the dosage may arise. The use of another type of medium, *e.g.* liquid, in which the bacilli, though still retaining their full virulence, could be obtained free from or at least could be easily washed free from the culture medium, particularly appeals to us. Regarding the variations in the reaction of rabbits of different breeds and from different localities to the test dosage we have been unable to find any reference in literature to work which would elucidate this point. We have also been unable to carry out the necessary work ourselves. During, however, the whole of the experimental work the rabbits have been obtained from the same supply agent, and have been frequently tested with known bovine and human types of the tubercle bacillus. Two

rabbits were always inoculated with bacilli from each strain. One rabbit received 0.01 mg. intravenously, and the other 10.0 mg. subcutaneously (between the shoulders). In five cases four rabbits were inoculated with each strain (two subcutaneously, and two intravenously), and in one case six intravenous tests and one subcutaneous were carried out. No discrepant results were obtained.

Our results are set out in Table I.

Table I.

Age period	No. of cases	Human type	Bovine type	Atypical
0-5 yrs.	7	7	0	0
5-10 yrs.	13	13	0	0
10-16 yrs.	15	12	3	0
Adults	8	8	0	0
Total	43	40	3	0

Table I relates to both glandular and bone and joint cases of tuberculosis. Table II refers only to bone and joint cases.

Table II.

Age period	No. of cases	Human type	Bovine type	Atypical
0-5 yrs.	7	7	0	0
5-10 yrs.	12	12	0	0
10-16 yrs.	14	12	2	0
Adults	5	5	0	0
Total	38	36	2	0

This gives a percentage of 6.0 due to infection by the bovine type of the tubercle bacillus in cases of bone and joint tuberculosis belonging to the age group 0-16 years.

Table III compares the observations of different observers, and relates only to cases of bone and joint tuberculosis.

Table III.

Investigator	District	Date	Age period (years)	No. cases	% Bovine
Fraser	Edinburgh	1912	0-12	67	65.6
Griffith, A. S.	Edinburgh	1916	0-12	19	26.3
Griffith, A. S.	Scotland	1916	0-16	25	32.0
Griffith, A. S.	England and Wales	1916	0-16	103	15.5
Eastwood and Griffith, F.	England and Wales	1916	0-16	217	23.9
Own cases	Sheffield	1924	0-16	33	6.0

In studying the results of such investigations it is absolutely essential to compare figures for the same age-groups. The technique employed is also important. With the exception of Fraser all the other observers mentioned in Table III have employed the same technique. Another matter of importance is that for percentage purposes large numbers of cases are essential. This was pointed out by both Eastwood and F. Griffith (1916), and by A. S. Griffith (1916) who noted that their "bovine" cases turned up irregularly. For this reason our relatively small number of cases may not give an absolutely accurate percentage for the Sheffield area; but in any case it is sufficient to

show that in this district the number of "bovine" infections in bone and joint tuberculosis in children is exceedingly low.

To study the cause of this it is helpful to compare the statistics of Sheffield with those of Edinburgh where the percentage of "bovine" infections has been found to be extremely high.

- The estimated amount of cow's milk consumed per person per diem
in Edinburgh = 0.42 pint,
in Sheffield = 0.27 pint.

In addition it may be noted that Mitchell (1925) states that in Edinburgh a large amount of the milk is consumed in the raw state. This is not so in Sheffield. From this one would gather that in Edinburgh the population has—other conditions being equal—a greater chance of becoming infected with tuberculosis through the milk supply.

2. Table IV gives the percentages of "mixed milk" samples which are infected with *B. tuberculosis*.

Table IV.

Years	Sheffield		Edinburgh	
	No. of samples	% positive	No. of samples	% positive
1902	28	17.8	—	—
1903	66	16.7	—	—
1904	89	6.7	—	—
1905	168	14.7	—	—
1906	115	9.6	—	—
1907	175	9.7	—	—
1908	251	9.9	—	—
1909	184	10.9	—	—
1910	336	10.4	—	—
1911	358	9.8	—	—
1912	447	7.6	—	—
1913	632	7.1	—	—
1914	595	7.2	—	—
1915	584	9.1	—	—
1916	365	6.6	406	20.0 (Mitchell, 1916)
1917	46	8.7	—	—
1918	48	6.3	59	8.4
1919	359	6.7	86	9.3
1920	836	7.0	81	9.9
1921	1057	8.3	86	11.6
1922	1073	7.5	91	2.2
1923	957	7.1	38	5.2
1924	966	7.5	57	14.0

The marked decrease in the percentage of positive "mixed milk" samples in Sheffield since 1902 is worthy of note. A further small reduction is likely under the new Tuberculosis Order of 1925 by which cows producing milk infected with the tubercle bacillus are compulsorily slaughtered. Beyond this, improvement can only be expected in proportion as the "tubercle-free" herds increase in number.

We feel that we cannot leave this particular subject without deploring the unpopularity of pasteurised milk. This appears to be chiefly due to what may be called the "Vitamine boom" in medicine. We do not for one minute wish to underrate the importance of vitamins; but may we ask whether it

is more important to escape the decided risk of being infected with tuberculosis and lose some of the vitamines principles, or *vice versa*, especially as these vitamines can easily be supplied in other foods? We should also like to refer to a statement which we have often heard expressed; in some cases, it is regrettable to note, by medical men, and which appears to us to be of a most pernicious character. It is to the effect that it is a waste of time to eliminate tubercle bacilli from the milk supply as the infected milk acts as a beneficial vaccine against tuberculosis. How futile it seems that some can advocate the vaccination of the public with an organism, undoubtedly lethal in sufficient dose, and then to leave that dose entirely to chance!

The following figures are instructive and show the effect of the consumption, to a great extent in the raw state, of a milk supply largely infected with the tubercle bacillus on the amount of "bovine" infection in cases of bone and joint tuberculosis in children.

Percentage of "mixed milk" samples containing tubercle bacilli
 in Edinburgh in 1916 = 20.0 per cent. (Mitchell, 1916),
 in Sheffield in 1924 = 7.5 per cent.

Percentage of "bovine" infections in bone and joint tuberculosis in children
 in Edinburgh in 1915-16 = 26.3 per cent. (A. S. Griffith, 1916),
 in Sheffield in 1923-24 = 6.0 per cent. (own figures).

The number of bone and joint cases of tuberculosis per thousand of population notified

in Edinburgh in 1915 = 0.281,
 in Sheffield in 1924 = 0.194.

As a rough guide to the amount of "human" infections in the population the respective cases of pulmonary tuberculosis notified per thousand of population are now indicated

in Edinburgh in 1915 = 2.133,
 in Sheffield in 1924 = 2.788.

The rate for cases of bone and joint tuberculosis per thousand of population notified in Edinburgh in 1924 was 0.185. This compares very favourably with the 1915 rate, *i.e.* 0.281, and a further study of this matter in Edinburgh would be of great interest.

CONCLUSIONS.

1. Neutralised inspissated serum is superior to the ordinary preparation for the growth, especially in primary cultures, of the tubercle bacillus.
2. Incubation at room-temperature is in some cases very useful in the differentiation of strains of the tubercle bacillus into their appropriate cultural groups, *i.e.* whether they are eugonic or dysgonic.
3. The amount of bone and joint tuberculosis in children in Sheffield due to infection with the bovine type of the tubercle bacillus is relatively low, *viz.* 6.0 per cent.

4. This is shown to be closely related to the relatively small amount of milk which is infected with the tubercle bacillus consumed by the inhabitants.

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