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CORRELATION OF THE SULPHITE REDUCTION TEST WITH OTHER TESTS IN THE BACTERIOLOGICAL EXAMINATION OF WATER.

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(With Plate I.)

IN previous papers (1922, 1924) we have investigated the capacity possessed by certain micro-organisms of reducing sulphites to sulphides, and have applied it (1) to the differentiation of bacteria, and (2) to the bacterioscopic analysis of water.

Since our last communication we have employed the sulphite reduction test in the examination of 252 specimens of water, most of which were sent to these Laboratories by Sanitary Authorities for report.

In our hands the test has proved most helpful and an analysis of our results is here presented. We may recall the fact that *B. typhosus*, *B. enteritidis* Gärtner, and certain members of the paratyphoid group form deep black colonies in a medium consisting of glucose agar in which are incorporated sodium sulphite and an iron salt, and that the great majority of the strains of *B. coli* are unable to bring about this reduction. This power is also possessed by the obligatory anaerobes of the intestine. We have found that *B. welchii*, *B. fallax*, *B. chauvoei*, *B. sporogenes*, *B. botulinus*, *B. parasporogenes*, *B. bifermentans*, and *B. sphenoides* produce dark colonies and that *B. tetani*, *B. tertius* and *B. histolyticus* cause the medium to turn greenish black, whilst *B. oedematiens*, *B. tetanomorphus*, *B. cochlearius* and *B. butyricus* do not change the colour.

If the reduction test is to be of any service in water analysis it must be shown that pure water free from excretal contamination does not contain any reducing micro-organisms and that contaminated water contains them in considerable numbers. Our experience bears out the truth of this contention. The origin of the anaerobes that are present in soil is in many cases unknown, but the researches of Klein, Houston, Thresh and others have demonstrated that *B. enteritidis sporogenes* Klein is a useful indicator of excretal pollution recent or remote. It is stated by Dubovsky and Meyer (1922) that *B. botulinus* has been met with in virgin soil, and the same may be true of other anaerobes, but, as regards water supplies, we have found by quantitative determinations of the anaerobes that their number increases almost *pari passu* with the pollution.

METHOD.

The method which was used throughout the examinations consisted in mixing 40 c.c. of the water with an equal volume of nutrient glucose agar medium containing 3 per cent. agar, to every 100 c.c. of which were added 1 c.c. of an 8 per cent. ferric chloride solution, 0·6 c.c. of a 10 per cent. sodium hydrate solution, and 10 c.c. of a 20 per cent. solution of sodium sulphite (anhydrous). These solutions were added to the freshly boiled and melted agar which was, whilst hot, poured into the water and then after admixture poured out into a large Petri dish. The cold water immediately cooled down the agar and prevented any risk of destruction of the bacteria by heat. After the medium had set it was covered over with a layer of the sulphite agar mixed with sterile water. This procedure was designed to protect the bacilli near the surface from the action of the oxygen of the air.

Plate I indicates the appearance of the reducing colonies. We classify the colonies into large (diameter 5 mm. or more), medium (3–4 mm.), small (1–3 mm.), and tiny (1 mm. or under). The colony of *B. welchii* is nearly always medium or large. That it is a *B. welchii* colony can be verified by removing it by means of a platinum wire, placing it in a small test-tube and filling the tube with milk which has been recently boiled and cooled. The characteristic "stormy" fermentation develops on incubation and gram-positive non-sporing bacilli are seen on microscopic examination.

Large dark colonies have almost always proved to be *B. welchii*, whilst the others are formed by obligatory anaerobes which we have not identified. In two or three instances we have found the dark colonies contained lactose fermenting *B. coli*. Such *B. coli* reducers are very rarely met with and, so far, we have failed to isolate any typhoid or paratyphoid bacilli when we made a special study of the reducing bacteria found in waters which were under suspicion in connection with outbreaks of enteric fever.

As the spores of *B. welchii* and the other anaerobes are very resistant, it is evident that it would be difficult to find any water so pure that an occasional micro-organism of this class might not be encountered—hence the importance of placing the test on a quantitative basis. It may be argued that the pollution indicated may be so remote that the water, though it does contain spores of intestinal anaerobes, is not even potentially dangerous. However, the number found is an important factor and in practice there is a fair degree of correlation between the *B. coli* and the sulphite test. If there are no reducers in 40 c.c. of the water it is very unlikely that such a water would convey disease as it shows freedom not only from recent but also from remote contamination. On the other hand, if there are numerous reducers and few *B. coli*, we would regard the supply with suspicion as there must have been a serious degree of contamination of the water with putrefactive bacteria probably of intestinal origin and this contamination may be of an intermittent kind and therefore liable to be repeated. For instance, on several occasions, in a small town the

sand filters gave irregular results, at one time a sample showing *B. coli* in 1/10 c.c. and at another time no *B. coli* in 20 c.c., but in both cases numerous reducing colonies were found in the tap water, no doubt due to the spores persisting in the slime of the pipes from the period when impure water was entering the system.

The variation in the number of reducing colonies in samples containing the same number of *B. coli* throws further light on the quality of such supplies. For instance, a well water showed *B. coli* in 0·2 c.c. but 59 reducing colonies in 40 c.c. This was found on enquiry to be from a well some fifty feet deep situated at the corner of a cemetery. Such a sample would be much more dangerous and objectionable than water from a well containing coliform bacilli in 0·1 c.c. and situated in a grazing field and which contained no reducers in 40 c.c. The test is a useful indicator not only of faecal pollution but also of pollution due to the decomposition of animal remains, where the *B. coli* test may not indicate how seriously the supply is contaminated. The bacteria responsible for decay of vegetable matter are not sulphite reducers. In the tables following, the results of the analysis of 252 samples of water are summarised in a form which attempts to correlate the findings of the *B. coli* and reduction tests.

CORRELATION OF *B. COLI* AND SULPHITE-REDUCING MICRO-ORGANISMS.

In our experience pure waters contain no reducing micro-organisms even in 40 c.c. of the water. Rivers derived from the clean valleys of the Mourne Mountains usually show none and never more than 1 or 2 colonies in 40 c.c. Similarly, waters derived from clean catchment areas and stored in reservoirs usually show only 1 or 2 colonies. On the other hand, polluted streams and dams show large numbers. There is a certain measure of agreement between the findings based on the *B. coli* test and on those of the reduction test. If we take as a standard in the reduction test that the black colonies of all kinds should not exceed 4 when 40 c.c. of the water are planted out, we shall see from the tables that shallow wells containing this number will also be condemned on account of the *B. coli* presumptive test being positive in 1 c.c. or less amounts.

When the presumptive *B. coli* test is positive with 1/10 c.c. it is usual to find reducing colonies ranging from 5 to 320 per 40 c.c. We have, however, not infrequently found no reducers present in 40 c.c., although the presumptive *B. coli* test was positive with 1/10 or 1 c.c. It is in these cases particularly that the reduction test has shown itself valuable as indicating the importance of ascertaining whether the organisms yielding acid and gas in MacConkey's lactose broth were really true excretal *B. coli*. In 20 instances where there was this divergence in the findings of the two tests, we found in all that the coliform bacilli which were present were not true *B. coli* but belonged to the *B. aerogenes* group.

As pointed out by one of us (1922) in a former paper we have found the

Source	Minimum amount of water in c.c. in which presumptive *B. coli* test was positive in bile-salt lactose broth	Number of samples	Number of dark colonies developing from 40 c.c. in glucose-sulphite-iron agar
Shallow wells	1/300	1	361
	1/100	3	29, 44, 127
	1/50	1	7800
	1/10	53	0*, 0*, 0*, 0*, 0, 0, 1, 1, 1, 1, 2, 2, 2*, 2, 2, 3*, 3*, 4, 4, 5, 6, 7*, 7, 10, 10, 10, 12, 13, 14, 14, 14, 15, 16, 16, 16, 18, 20, 26, 26, 33, 33, 40, 61, 62, 64, 66, 68, 81, 84, 94, 155, 253, 320
	1/5	5	4, 25, 35, 59†, 72
	1	30	0*, 0*, 0*, 0*, 0*, 1*, 1, 2*, 2*, 3, 3, 3, 4, 4, 4, 6, 9, 10, 11, 14, 16, 16, 17, 18, 24, 27, 27, 72, 82, 89
	3	7	0*, 0, 1, 2, 5, 2, 6
	5	8	0*, 0, 1*, 6, 6, 9, 18, 22
	10	10	0*, 0*, 1, 2, 2, 3, 4, 5, 7, 83
	20	3	0, 1, 6
	40	2	0, 7
	50	2	1, 1
	80	1	0
	B. coli absent from 50	5	0, 0, 0, 0, 2
	B. coli absent from 70	2	0, 1
	B. coli absent from 100	1	0
Streams and rivers	1/2000	1	182 (spores)
	1/500	1	174
	1/200	1	568
	1/100	1	256
	1/80	1	276
	1/10	10	4, 20, 32, 59, 71, 71, 77, 139, 160, 178
	1/5	2	16, 30
	1	6	3, 3, 7, 15, 17, 22
Reservoirs and dams	1/10	3	8, 37, 57
	1/5	2	8, 84
1. *Unfiltered*	1	8	0, 1, 1, 1, 1, 1, 2, 9
	3	9	0, 1, 1, 1, 3, 5, 9, 9, 14
	5	5	1, 1, 4, 18, 20
	10	3	0, 2, 3
	20	2	1, 2
	40	1	5
	50	7	0, 0, 0, 1, 1, 6, 11
	80	2	1, 4
2. *Filtered*	1/10‡	4	1, 2, 4, 4
	1	7	0, 0, 0, 0, 0, 0, 2
	3	1	0
	5	2	0, 2
	10	1	3
	20	1	0
	30	4	0, 0, 0, 1
	50	8	0, 0, 0, 0, 0, 1, 1, 1
	60	1	0
	80	5	0, 0, 0, 2, 1
Springs	1/10	2	1, 2
	1	7	1, 1, 4, 4, 5, 6, 101
	5	1	1
	10	1	0
	20	1	0
	B. coli absent from 40	1	0
	B. coli absent from 50	4	0, 0, 1, 3
	B. coli absent from 60	2	0, 0

* Coliform organism present in presumptive test was proved to be *B. aerogenes* by the methyl-red, Voges-Proskauer and Koser tests.

† Well in cemetery.

‡ Filters defective.

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methyl-red and Voges-Proskauer and Koser's uric acid tests useful in the differentiation of excretal *B. coli* from *B. aerogenes*. More recently we have employed a new method introduced by Koser (1923) which has proved most helpful. This new medium of Koser (citrate medium) has the following composition: Distilled water 1000 c.c., NaCl 5·0 gm., $MgSO_4$ ($7H_2O$) 0·2 gm., $(NH_4)H_2PO_4$ 1·0 gm., K_2HPO_4 1 gm. and 2·77 gm. sodium citrate ($5\frac{1}{2}H_2O$).

Like Koser, we have found that *B. coli* isolated from faeces or colon bacilli in water supplies which were probably of excretal origin fail to grow in the medium, being unable to utilise sodium citrate as a source of their carbon, whereas *B. aerogenes* and certain coliform bacilli found in water supplies are able to cloud the medium. In our opinion such organisms are probably found in water and soil apart from excretal contamination. When we have none or very few reducers in 40 c.c. of the sample and yet the presumptive *B. coli* test positive with 5, 1 or even 0·1 c.c., we have almost invariably found that the coliform bacillus could grow in Koser's citrate medium and was probably a resistant organism adapted to life in the outer world.

Another useful test and one which has been strongly advocated by Savage and by Wood (1919) is the determination of the presence or absence of streptococci. It is only recently that we have examined for streptococci as a routine procedure, but a determination of their presence or absence in 10 c.c. of water promises to be a useful adjuvant to the *B. coli* and reduction tests. We have found the following medium to be exceedingly useful in obtaining cultures of *streptococci* and *pneumococci* from sputum and uterine swabs, urine, etc., and were encouraged to apply it to water analysis. The medium as used by us in clinical pathology consisted of equal parts of ordinary nutrient broth and of Clark and Lubs medium, the latter consisting of 0·5 per cent. each of peptone, di-potassium hydrogen phosphate and glucose in distilled water. In water analysis we use 10 c.c. of double strength medium and to it add 10 c.c. of the water to be tested. The deposit in tube after 18 hours' growth at 37° C. is taken up by a capillary pipette and films stained by Gram's method using weak carbol-fuchsin as counter-stain.

RELATIVE FREQUENCY OF *B. WELCHII* AMONGST THE BLACK COLONIES.

The colony of *B. welchii* is nearly always large or medium in size. Occasionally, where there are enormous numbers of *B. coli* present, the growth of the latter interferes with the growth of the former and in consequence the area of dark colour is not so large. In a faecal emulsion which has been heated to 80° C. for 10 minutes the number and size of the dark colonies is frequently greater than with an unheated specimen.

In many samples of water all the dark colonies are found to consist of *B. welchii* whilst in others perhaps only 1/10 of them contain this bacillus. In 27 consecutive samples examined there was a total of 726 dark colonies developed from $27 \times 40 = 1080$ c.c. of the waters and these were classified as follows: 158 "large," 240 "medium," 102 "small," and 226 "tiny." In the

following table the results of the application of the milk test to the dark colonies encountered in another set of samples are given:

Total	Large l.	Medium m.	Small s.	Tiny t.	Number examined	"Stormy" fermentation	Not "stormy"
6	6	—	—	—	6	6	0
33	32	1	—	—	27	24	3
16	0	7	9	—	8	4	4
4	0	2	2	—	2	1	1
77	42	20	15	—	15	8 (7 l., 1 m.)	7 (1 l., 2 m., 4 s.)
101	16	51	34	—	14	4 (4 l.)	10 (4 l., 4 m., 2 s.)
7	5	0	2	—	6	1 (1 l.)	5 (3 l., 2 s.)
20	—	—	—	—	6	4 (2 l., 2 m.)	2 (1 l., 1 s.)
18	—	—	—	—	5	2 (1 l., 1 m.)	3 (2 m., 1 s.)
2	—	1	1	—	2	0	2
1	—	1	—	—	1	0	1
4	2	1	1	—	4	1 (1 l.)	3 (1 l., 1 m., 1s.)
33	16	13	0	4	16	12 (9 l., 3 m.)	4 (1 l., 2 m., 1 t.)
1	1	—	—	—	1	1	—
1	1	—	—	—	1	1	—
4	4	—	—	—	4	4	—
25	4	20	1	—	12	0	12 (4 l., 7 m., 1 s.)
16	1	12	3	—	10	7 (1 l., 5 m., 1 s.)	3 (3 m.)
2	2	—	—	—	2	2	—
3	1	2	—	—	3	1 (1 l.)	2 (2 m.)
29	—	14	15	—	12	9	3
14	—	—	—	—	13	2	11
35	28	7	—	—	35	28 (24 l., 4 s.)	7 (4 l., 3 s.)
14	4	6	0	4	7	4 (4 l.)	3 (3 m.)
3	1	—	—	2	1	0	1 (1 l.)
2	2	—	—	—	2	1 (1 l.)	1 (1 l.)
27	22	1	0	4	19	17 (17 l.)	2 (2 l.)
5	1	2	—	2	5	3 (1 l., 2 m.)	2 (2 t.)
					239	147	92

We see from this table that of 239 colonies examined, 147, or approximately 60 per cent., gave "stormy" fermentation of milk and, in the whey, gram-positive non-sporing bacilli resembling *B. welchii* were found. It is probably not very wide of the mark to state that of the dark colonies encountered in several hundreds of samples of water half of them would be classified as large or medium and of these about 50 per cent. would be found to be *B. welchii*. Probably 1 in every 4 of the dark colonies is composed of *B. welchii*.

Our view is that all the dark colonies whether tiny or large are formed by intestinal bacteria. It may be that the qualitative differentiation of these anaerobes would be helpful and that the number of true *B. welchii* colonies should be ascertained since this bacillus has been recognised for many years as a useful indicator of faecal pollution. However, the principle of the test as at present constituted, is to determine the number of sulphite reducers and these we know are nearly all obligatory anaerobes, though occasionally a facultative anaerobe such as certain strains of *B. coli* or of the typhoid-para-typhoid group may be encountered.

In France it is customary in water analysis to determine *l'indice anaérobique, i.e.* the proportion between the true anaerobes and the aerobes. The test is carried out in long tubes filled with glucose-gelatin tinted with sulphindigotate of soda. It is stated that the colonies of the true anaerobes can be

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distinguished by their appearance and the bleaching of the medium from those that are facultative.

Guillemard (1906) and Vincent (1907) appear to have introduced the test and it is referred to by Calmette (1925) as one that is with advantage commonly meployed.

As regards a bacteriological standard in dealing with *B. enteritidis sporogenes* in water supplies, the following quotation from Sir Alexander Houston (1901) is pertinent: "The spores of *B. enteritidis sporogenes* are present in 1/100 to 1/1000 c.c. of sewage. In virgin soils this microbe has a sparse distribution, but in polluted and cultivated soils it is present in great abundance (1000 to 10,000 per gramme). Pure water may contain no spores even in 100–500 c.c. or more of the sample. Speaking in general terms a reasonable view would seem to be this—a potable water should be condemned if it contains *B. enteritidis sporogenes* in 10 c.c. and regarded with suspicion if this microbe is present even in 10 c.c. Absence of *B. enteritidis sporogenes* from 100 c.c. implies relative safety, but absence from even 200 c.c. need not necessarily in all cases be accepted as indicating absolute freedom from danger of a potential if not actual kind."

From Houston's extensive investigations we know that in crude sewage 100,000 *B. coli* and 100 to 1000 spores of *B. enteritidis sporogenes* are usually found in each cubic centimetre. In contaminated water the resistant character of the spores compensates for their smaller numbers.

Thames water at Hampton and Sunbury has been found by Houston frequently to show *B. coli* in 1/10 c.c. and *B. enteritidis sporogenes* in 10 c.c. Here we may point out that *B. enteritidis sporogenes* Klein (1895) is undoubtedly identical with *B. aerogenes capsulatus* Welch and Nuttall (1892). The differences between the descriptions given by their respective discoverers can be explained on the ground that Klein had in his cultures not only *B. aerogenes capsulatus*—now usually termed *B. welchii*—but also *B. sporogenes* Metchnikoff.

For routine work 40 c.c. of water is sufficient to examine by the reduction test. In examining for *B. enteritidis sporogenes* in the past it was usual to concentrate the spores by filtration through a porcelain filter. This involves a good deal of labour and sedimentation of the bacteria by the addition of alum probably gives as good results. We find that the coagulum of aluminium hydrate can be incorporated in the glucose-sulphite-iron-agar medium and that it does not interfere with the growth of the dark colonies.

TESTING WATER FOR *B. TYPHOSUS*.

The fact that the typhoid bacillus and various members of the paratyphoid B group differ from the great majority of colon gram-negative bacilli in their capacity to reduce sulphites suggests that use might be made of this fact in the search for typhoid bacilli in suspected water supplies. We find that it is possible to recover typhoid bacilli which have been added in small numbers

to the water, by means of alum precipitation and then planting out in glucose-sulphite-iron-agar. Under these circumstances it is necessary to suppress as far as possible all other reducing bacteria. So far, we have not succeeded in suppressing a few strains of *B. coli* which are met with in small numbers in faeces but rarely in water supplies, but we have devised a means of preventing the growth of *B. welchii* and other intestinal anaerobes. This consists in the addition of Brilliant Green to the medium.

The effect of the presence of sodium sulphite is to reduce the antiseptic action of Brilliant Green for all bacteria, but in the sulphite medium in a concentration of 1 in 1000 to 1 in 20,000 Brilliant Green allows the growth of *B. typhosus* but inhibits that of *B. welchii* and other anaerobes.

To 100 c.c. of sulphite-glucose-iron-agar medium, 2 c.c. of a 1 per cent. watery solution of Brilliant Green is added giving a concentration of 1 in 5000 and, when the medium is mixed with an equal volume of water, a concentration of 1 in 10,000. The black colonies can be easily planted out on MacConkey's lactose bile-salt-neutral-red-agar and agglutination and other tests applied.

Conclusions.

1. When 40 c.c. of water samples are added to an equal volume of a glucose-sulphite-iron-agar medium, the number of dark sulphide colonies varies from 0 to several hundreds according to the degree of contamination of the water.

(*a*) Very pure water, whether surface or well, shows none.

(*b*) A potable water should not show more than 4.

(*c*) Sand-filtered water should not show more than 1.

2. There is a fairly close agreement in the results of the *B. coli* and the reduction tests. Where *B. coli* is found in small amounts of water, of which 40 c.c. contain no reducers, it has been found that the coliform bacilli are not true *B. coli* but that they are allied to *B. aerogenes* as shown by Koser's citrate test.

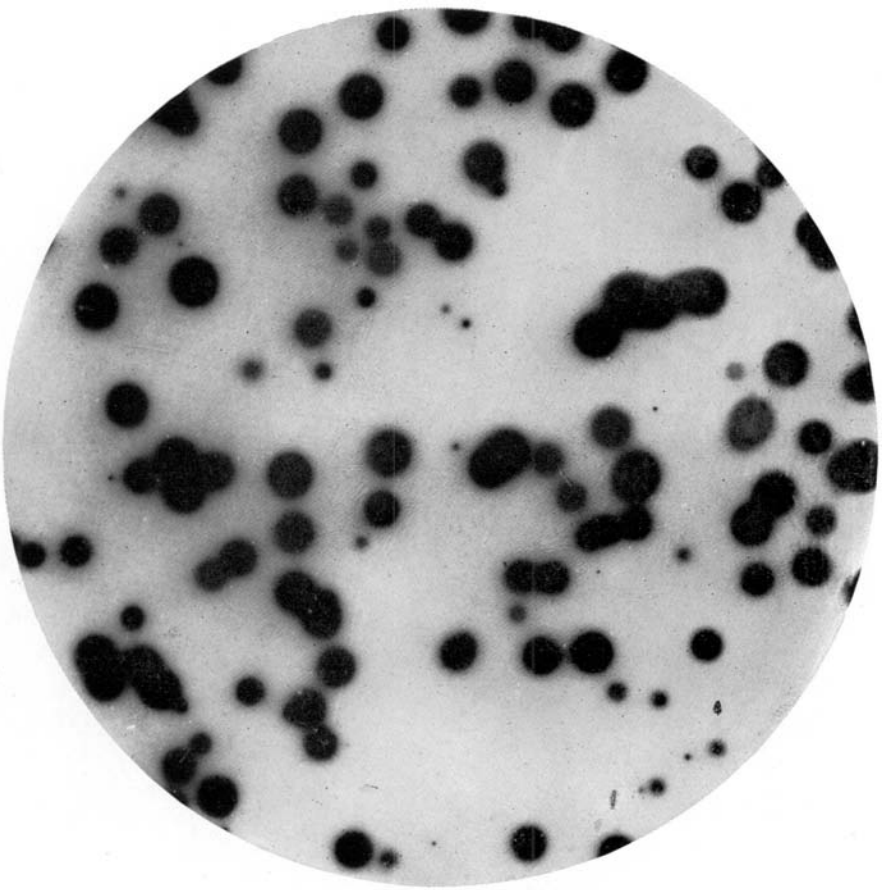
The dark colonies can be classified according to their size into large, medium, small and tiny.

B. welchii usually forms a large or medium colony.

3. A selective medium for *Streptococci* is described.

4. The sulphite medium can be applied in attempts to isolate typhoid bacilli from water or stools, the addition of Brilliant Green in 1 in 1000 to 1 in 20,000 inhibiting the growth of anaerobes whilst allowing that of *B. typhosus* in the sulphite medium.

5. The results obtained in the examination of 252 samples of water by the *B. coli* and sulphite reduction tests are correlated.



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DESCRIPTION OF PLATE I.

Photograph of growth in a glucose-iron-sulphite-agar plate inoculated with 40 c.c. of water taken from a contaminated stream.

Large, medium, small and tiny colonies are seen.

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