NOTE ON THE *B. COLI* TEST.

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My friend Major Wesley Clemesha, I.M.S., in a recent contribution[1] to the *Journal of Hygiene* is good enough *inter alia* to say:

> "It may be safely stated that anything which Dr A. C. Houston writes on the subject of water bacteriology is received with very great interest and respect, and his recent report on the bacteriological characters of *Bacillus coli* in raw, stored and filtered river water (*Metropolitan Water Board, 7th Report on Research Work*) is no exception to the rule. The report is of very great interest to all engaged in the study of water bacteriology, particularly in England."

In attempting, however, to answer some of Clemesha's criticisms, it will be understood that I do so in a spirit the reverse of hostile. My position coincides with my inclination, and precludes me from entering the arena of controversy. Destructive criticism is nearly always to be strongly deprecated, and any remarks that I have to make are offered from the point of view of the constructive critic who is anxious to clear up any misconceptions and to bring into harmony views which may be apparently rather than really divergent.

The following quotations are taken from pages 463–465 of Clemesha's paper.

[1] Clemesha, W. W. (10. I. 1913), "A Criticism of Dr A. C. Houston's Report on the Biological Characters of *B. coli* Isolated from (1) Raw, (2) Stored River Water, (3) Stored and Filtered Water." *Journal of Hygiene*, XII. pp. 463–478.

Note:—To assist the reader, quotations from Clemesha's paper are printed in ordinary type; quotations from my own reports appear in smaller type; both are indented.

https://doi.org/10.1017/S0022172400005490 Published online by Cambridge University Press

Dr Houston commences his report as follows:

"For many years past I have pointed out the striking differences between the varieties of *B. coli* found in excremental matter and in waters of diverse origin. As regards the Metropolitan water supply, it has been shown that the proportion of the *B. coli* organisms which ferment lactose with gas production (lactose +) and also produce indol in peptone cultures (indol +) as compared with those which fail in one or other or both of these respects, is as follows:

Water	Samples	Specimens (Glucose+)	Typical "Lactose+ Indol+" (per cent.)	Non-typical "Lactose − Indol+" or "Indol − Lactose+" or "Indol − Lactose −" (per cent.)
Raw River Waters	3,546	12,744	81·2	18·7
Filtered Waters	35,696	18,960	50·8	49·1

"These results have always been, and are now again, given simply as facts observed, when working with liquid primary media and not, as in any way, necessarily representing the actual ratio of typical to non-typical *B. coli* in the water originally.

"These findings have resulted from the adding of 100 c.c., 10 c.c., 1 c.c. and lesser amounts of water to one or other liquid primary medium, incubating at or about blood heat, usually for forty-four hours, planting out the colonies on one or another solid medium, and picking off the suitable colonies, and studying their biological attributes in a variety of culture media.

"Two points are specially to be remembered in connection with the use of liquid primary media. (1) The original proportion between the various kinds of *B. coli* may be greatly altered by the multiplication process. Indeed, the method aims at encouraging the typical *B. coli* to multiply and oust the non-typical *B. coli* in the struggle for existence in a liquid nutrient medium. (2) The specimens of *B. coli* may and do pertain to different volumes of the water being tested."

Clemesha goes on to say:

"In the quotation given above Dr Houston explicitly says that the figures obtained do not necessarily represent the actual ratio of the two kinds of bacilli in the original water. Now supposing that it can be definitely proved that there is a very great difference in the ratio of the classes between the actual water and these results, surely it must be admitted that the method is unsatisfactory. In other words, any method which can be proved to give a really reliable picture of the flora actually present in the water sample, must be superior, from the point of view of the water analyst, to one which cannot be relied on to do this. The main object of all analyses of water is to obtain an accurate picture of the actual bacteriological state of affairs in the sample at the time, so that if

'the original proportion of the various kinds of coli may be greatly altered by the multiplication process,'

the utility of this particular method appears to us to be very questionable. For many years Dr Houston has been using this 'enrichment' method and presumably has been basing his opinion of the quality of water on its results; probably most water analysts besides ourselves believe that, when using a method very similar to his, they are getting a reasonably accurate picture of the flora of the sample in question so that it comes rather as a shock to us in India to read that Houston now says that this is not the case.

Dr Houston not only says that

'the original proportion between the various kinds of *Bacillus coli* may be greatly altered by the multiplication process" (in broth media) but "the method aims at encouraging the typical *Bacillus coli* to multiply and oust the non-typical in the struggle for existence in a liquid nutrient medium.'

In the first place, we wish to say that, within certain limits, we very much doubt the accuracy of both these statements, and, secondly, we should like to know on what experimental evidence this favouring of one variety of organism at the expense of others is actually based. No evidence of this kind is given in the report."

It is to be noted that I did not say that the original proportions between the various kinds of *B. coli* "*is*" greatly altered by the multiplication process, but "*may be*" greatly altered, etc. Also the words "do not *necessarily* represent, etc." were employed purposely in place of the words "*do not represent*," etc.

Is not Clemesha going a little too far when he says:

"The main object of all analyses of water is to obtain an accurate picture of the actual bacteriological state of affairs in the sample at the time, so that if 'the original proportion of the various kind of *coli* may be greatly altered by the multiplication process' the utility of this particular method appears to us to be very questionable."

Personally, I think that we obtain the truest hygienic picture, by purposely using media and methods, which do in effect tend to alter the (initial) proportion of microbes in favour of those whose presence we have come to regard as specially significant of undesirable pollution. I trust the shock to my friends in India will be mitigated when I

assure them that I still remain a firm believer in the "enrichment" method, for the very reason that I believe it tends to ensure the subordination of the bacteria which occupy a secondary place, as indicators of potential danger, and the exaltation of those of more direct importance.

It may be of interest to look at the direct and indirect (enrichment) methods from the point of view of a person possessed of no special bacteriological knowledge.

I venture to think that the following analogy is a fair one:

It is sought to ascertain whether there are any fish in a fish pond, and if so what species are present, and their relative abundance.

The fish pond is divided up into a series of intercommunicating compartments of equal size which can if necessary be isolated from each other for draining or cleaning operations.

Direct Method.

We might select one of these compartments (corresponding, let us say, to our 1 c.c. cultures), drain it dry, and exhaustively count the fish. Imagine that we found a total of 100 fish made up of 5 Chubb, 15 Carp, 20 Bream and 60 Roach.

On the assumption that our compartment was a truly representative one we should then be in a position to affirm absolutely that the pond contained:

Chubb	5 %
Carp	15 %
Bream	20 %
Roach	60 %

Indirect Method.

We might elect to run the water *plus* all the fish from one of the compartments into a separate pond altogether (enrichment medium) one containing to start with, no fish of any kind. After one or more years (corresponding to incubating our cultures for 18–48 hours) we might net a *relatively very small portion* of it, corresponding to the very minute quantity we spread over our plates in making cultures from our primary liquid enrichment medium.

Imagine that our net captured exactly 100 fish should we be privileged on an investigation of their "species" to say our original pond necessarily contained this, that, or the other fish in the same proportions.

I think not, because the conditions in the second pond might be favourable to certain species and unfavourable to others. In much the same way, it seems to me that the direct method is almost necessarily and fundamentally the standard by which we must judge all enrichment methods, which seek to represent the "actual ratio of typical and non-typical *B. coli* in the water originally."

There may sometimes be technical difficulties which render the direct method difficult, perhaps almost impracticable, but this does not really affect the principle involved. I am not arguing against the "enrichment" method or in favour of the "direct" method but merely trying to show that whilst one judges the hygienic quality of a water best by the former method, the latter method *does*[1] (whereas the former *may* not) give the actual ratios of different microbes in the water originally.

The following experiment will serve as an illustration.

Equal volumes of a sample of river Thames water were used to inoculate:

(*A*) Solid lactose bile salt peptone agar plates (direct method).

(*B*) Liquid lactose bile salt peptone tubes (indirect enrichment method).

After 24 hours incubation at 37° C. 50 colonies were sub-cultured from *A*.

After (*a*) 24 hours and (*b*) 48 hours incubation at 37° C. solid lactose bile salt peptone agar plates were made from *B*.

After 24 hours incubation at 37° C., colonies were picked off from the *B* plates ((*a*) and (*b*)) in the same way and in the same number as had previously been done in the case of (*A*).

The following table shows the results obtained:

	A	B	
	Direct solid method	Indirect liquid enrichment method	
		(*a*)	(*b*)
Glucose 0	15	4	1
Glucose + Lactose 0	29	11	3
Glucose + Lactose +	6	35	46
	50	50	50

[1] It is assumed, of course, that all the colonies growing on the direct plates are subcultured, and a similar number subcultured from the indirect plates, the same volume of water being used in each case, and in sufficient amount so as to be representative.

https://doi.org/10.1017/S0022172400005490 Published online by Cambridge University Press

As for practical reasons only a proportion and not the whole of the colonies appearing on the *A* plates was subcultured it cannot be said that the results under *A* necessarily represent the whole of the facts. It seems to me, however, fairly obvious that the effect of the enrichment medium was to alter the initial ratios so as greatly to increase the relative number of lactose as compared with glucose fermenters.

This indeed is the chief merit of the enrichment process.

On page 468 Clemesha says:

> "Again, if a mixture of two bacilli, such as *B. coli communis* Escherich (which is lactose + glucose +) and our bacillus *P* (which is glucose + lactose −) are mixed together in roughly equal proportions and inoculated into a glucose broth, it can be proved that within the first 24 hours there is little or no alteration in the relative numbers of the two classes; both bacilli fermenting glucose. But if the same mixture be inoculated into lactose broth, the lactose fermenters (*B. coli communis*) very rapidly overgrow the bacillus *P*, which does not ferment the sugar. Even in this case white colonies are usually found in the plate. As one would expect, the more bacilli *P* there are in the original mixture the less is the overgrowth of *B. coli* apparent, but in cases where the numbers are approximately equal at the time of inoculation, the *B. coli communis* undoubtedly very rapidly outgrows bacillus *P*. This is after all only what one would expect—a bacillus that does not ferment lactose would probably grow slower in a lactose medium than one that does—indeed it seems to us rather astonishing that a non-fermenter should grow so well in a sugar that it cannot alter in any way."

These remarks would seem indeed to lend support to my guarded statement that:

> "These results have always been, and are now again, given simply as facts observed, when working with liquid primary media and not, as in any way, necessarily representing the actual ratio of typical to non-typical *B. coli* in the water originally."

Clemesha goes on to say:

> "Another series of experiments has been carried out in order really to ascertain whether the picture obtained from subculturing an 18-hour broth culture is reasonably like the original substance used for inoculation. A small piece of faeces of either human

being or cow, was taken and was divided into two parts. One was carefully wiped over 4 or 5 bile salt neutral red lactose agar plates. The other was inoculated into a bile salt lactose broth, and was incubated for 18 to 20 hours (inoculation was usually made in the afternoon and subcultured on the following morning). A careful comparison between a large number of colonies obtained by both methods, shows that the results are practically identical, the species of coliform organisms and their numerical relation to each other correspond to a most wonderful degree. The more colonies that are identified in each case the greater is the resemblance between the two results. For our experiments we took 50 colonies from both and we carefully identified each organism.

It will be observed that these experiments are infinitely more conclusive than those quoted by Dr Houston making use of raw, stored and filtered water. The possibilities of error in centrifugalisation, precipitation, evaporation, filter-brushing, etc., are extremely great: whereas in our experiments making use of faeces, all these very doubtful factors are eliminated."

I believe the reason why the results by the direct and indirect methods corresponded so closely in the above experiment was because faeces and not water was used. Faeces contain typical *B. coli* in enormous numbers and I should have expected a closer parallelism between the results yielded by the two methods when working with this substance than with any other material. In the case of water the non-typical *B. coli* may greatly out-number the typical *B. coli* and this in my experience may lead to very different results being obtained.

Clemesha proceeds as follows:

"Dr Houston's method of water analysis and our own are almost identical in the first stage. For crude waters we inoculate both a lactose and a glucose broth with identical quantities of the sample, in order to find out the relative number of glucose and lactose fermenters present in the water. The full reasons for this procedure cannot be entered into here. For filtered waters we use a lactose broth only. Having done this, we take the tube that has received 20 c.c. of the sample, after 18 hours' growth in the incubator, and subculture the organisms present in this tube *only*. The various species of coliform organisms present are separated by MacConkey's method. We maintain that by this procedure we get

a very accurate picture of the true bacteriological flora of 20 c.c. of the water under analysis, and it is not necessary to separate species in smaller quantities. The actual number of lactose or glucose fermenters present in all smaller quantities is ascertained by the first step."

Personally, I prefer to make sub-cultures from *all* the tubes (100, 10, 1, ·1, etc. c.c.) yielding positive presumptive results, but I am far from suggesting that Clemesha's modified procedure is not well suited for the purposes he has in view and the kind of waters he is called upon to examine.

I should have liked to have been able to test the matter with the London filtered waters, but happily for the consumers, they are relatively so good that a direct culture with 20 c.c. of water spread over 20 plates would (generally speaking) yield either no colonies at all or too few for subcultural purposes.

The following instructive experiment has however been carried out:

Direct Method.

(*A*) 5 c.c. of river Thames water were spread over five solid lactose bile salt peptone agar plates. The plates were incubated at 37° C. for 24 hours and then *all* the colonies (47 in number) were submitted to cultural tests.

Indirect Enrichment Method.

(*B*) 5 c.c. of the same sample of water were added to liquid lactose bile salt peptone water and incubated for 18 hours at 37° C. Solid lactose bile salt peptone agar plates were made therefrom and these were then incubated for 24 hours at 37° C.

A suitable representative plate was then taken and a section marked off with a coloured wax pencil, so as to embrace 47 unselected colonies and *all* of these were admitted to cultural tests.

The following results were obtained (p. 401).

It is difficult to see how any other conclusion can be reached than that the *A* (direct) method here shows as nearly as possible the absolute ratio of the microbes capable of growing on bile salt agar and pertaining to 5 c.c. of the sample of water, because *all* the colonies (47 in number) growing on the plates were subcultured. As regards the *B* (indirect) method it is obviously impossible to subculture the thousands or

Indol	Glucose	Lactose	Saccharose	Dulcite		A (Direct)	B (Indirect)
0	0	0	0	0		35	5
0	+	0	0	0		1	0
0	+	0	+	+		1	0
+	+	+	+	+		1	0
0	+	0	+	0		2	0
+	+	+	0	0		3	7
+	+	0	0	0		1	20
0	+	+	+	0		2	1
+	+	+	+	0		1	9
+	0	0	0	0		0	4
0	+	+	0	0		0	1
					Total	47	47

millions of microbes resulting from the multiplication of those originally present in the water, but it seems a fair means of comparison to map out a section of a plate so as to embrace 47 adjacent unselected colonies and subculture all the colonies within that area.

What is quite certain is this, that if *A* be regarded as the standard, *B* presents a highly distorted picture of the original state of the water. The results of this experiment, especially as they confirm my previous experience, seem to me fully to justify my use of the words, "The original proportion between the various kinds of *B. coli* may be greatly altered by the multiplication process." It is at once admitted that Clemesha by altering his procedure so as to shorten the period of incubation tends in the direction of preserving the initial ratios. On the other hand it must be remembered that this very circumstance may possibly weaken the value of the test from the hygienic point of view. Unless my memory is at fault, MacConkey, a good many years ago, advocated incubation for 48 hours for the very reason that it tended to alter the initial ratios and to bring into prominence those microbes specially significant of undesirable pollution. It is not however suggested that Clemesha is in any way wrong in adopting the particular methods and procedure which in his experience yield the most useful results in India.

On pages 470–472 Clemesha endeavours to show that the results obtained by me by the "direct" and "indirect" methods are strikingly similar. It would occupy too much space to go into this matter in detail, but I should welcome confirmation of such a conclusion, because my records by the "indirect" method are very numerous indeed whereas those obtained by the "direct" method are by *comparison* few in number.

From the tone of Clemesha's whole paper I rather gather that he thinks that my investigations by the "direct" method aimed at showing the imperfections of the "indirect" method. This, however, is far from being the case, my real object being to disarm criticism by using both methods, as otherwise it might be said that I had made no attempt to study the subject by the "direct" method.

Is not Clemesha a little severe when he criticises my statement:

> "Lactose + indol + microbes are typical of excrement inasmuch as they are present therein in enormous number and are the predominant microbes in this material.
>
> "Waters not recently contaminated with excremental matters contain none or very few of these bacteria."

as follows:

> "To the second sentence we must take very grave exception. Waters are not infrequently met with in India, in lakes and storage reservoirs, which we know have received no pollution for probably two, three or even six months, yet the majority of these may contain as many as 1 to 10 lactose + indol + organisms per c.c."

Excremental matters usually contain 100,000 *B. coli* per gramme and even if a water contained 1 or even 10 *B. coli* per c.c. this is a very small number in comparison. A reference to my reports on storage, etc., would seem to justify my statement.

The remainder of Clemesha's paper is chiefly devoted to an expression of the desirability of differentiating between faecal organisms of separate species and applying the results obtained to the history of waters in relation to pollution. No one will quarrel with this view, and if some of us feel that the results so far obtained are in their practical bearing a little disappointing, it would be ungenerous not to applaud the work already done in this field of enquiry or to say anything likely to discourage the hope that work in the future will bear more ample fruit.

It is impossible, perhaps even undesirable, for all bacteriologists to see eye to eye with each other in considering and interpreting biological problems of considerable perplexity; but this need not debar us from displaying a tolerant attitude when dealing with the work of others. Otherwise we are apt, almost unconsciously, to slip backwards into the ranks of the mere destructive critic.