THE EFFECT OF ANAEROBIC SPORE-BEARING ORGANISMS ON THE VALIDITY OF THE PRESUMPTIVE COLIFORM TEST AS USED IN THE BACTERIOLOGICAL EXAMINATION OF WATER

By THE PUBLIC HEALTH LABORATORY SERVICE WATER SUB-COMMITTEE*

INTRODUCTION

The medium commonly used in this country for the presumptive coliform test is neutral-red bile-salt lactose peptone-water (MacConkey broth). Various quantities of water are inoculated into tubes of this medium and the tubes are incubated at 37° C. for 48 hr. (see Ministry of Health Report, 1939; Taylor, 1949). During and at the end of this period each tube is inspected for lactose fermentation, as shown by the production of acid and gas. The presence in any tube of acid and sufficient gas to fill at least the concavity of the inner inverted (Durham) tube constitutes a presumptive positive coliform reaction, the presumption being that the tube contains coliform bacteria which were entirely responsible for the fermentation and which could, if isolated, reproduce the result. The presence in the tube of coliform bacteria unable to produce adequate gas within 48 hr. at 37° C. is disregarded.

The accuracy of the presumptive positive reaction can be tested by spreading a small loopful from the tube on MacConkey agar and incubating overnight at 37° C. The various coliform colonies (whether red, pink or pale pink) appearing on the plate can then be individually tested for gas-production by inoculating each of the selected colonies into a fresh tube of MacConkey broth and incubating at 37° C. The production of adequate gas within 48 hr. by any of the final pure cultures confirms the original presumption.

Most presumptive positive tubes give a positive result to such a confirmatory test. In fact, most tubes yield excellent coliform colonies which on subculture in a fluid medium produce adequate gas after only overnight incubation. For this reason routine water examination has come to rely largely on the unconfirmed presumptive test as though it were specific for coliform bacteria. In those laboratories, however, where a confirmatory procedure is carried out, the presence of false presumptive reactions has been noted, i.e. presumptive positive tubes from which no coliform bacteria can be isolated. Although in any one laboratory the false presumptive reactions form only a small proportion of the total fermented primary tubes, their incidence in certain types of water, e.g. chlorinated water,

* The P.H.L.S. Water Sub-Committee is composed of the following members of the P.H.L.S. Staff: R. D. Gray, M.D., D.P.H. (Chairman); W. H. H. Jebb, M.A., M.D.; J. H. McCoy, M.B., B.Ch., D.P.H.; J. Morrison Ritchie, M.A., M.B., Ch.B., D.P.H.; A. J. Kingsley Smith, B.M., B.Ch.; and Joan M. Watkinson, B.Sc.; together with: E. Windle Taylor, M.A., MD., D.P.H., Deputy Director of Water Examination, Metropolitan Water Board, London; and Ian Sutherland, M.A., D.Phil., of the Medical Research Council's Statistical Research Unit.

may be so high as to give an entirely misleading impression of the sample, if reliance is placed solely on the unconfirmed presumptive test.

For this reason alone, it was felt that the work of previous investigators might be usefully analysed and extended and a further inquiry be made into some of the common causes of false reactions and the possibility of adapting the current minimum routine so as to distinguish false from true coliform reactions.

PRACTICAL DEFINITION OF A COLIFORM ORGANISM

An essential preliminary, however, is a clear and accurate understanding of which organisms, for the purpose of water examination, are to be regarded as coliform bacteria. Although for many years it has been agreed that the time limit for incubation of the primary tube shall be 48 hr., no similarly clear-cut time limit has yet generally been agreed upon for the incubation of the confirmatory tube. Thresh, Beale & Suckling (1943), Taylor (1949) and Hoather (in a personal communication) recommend the limit of 48 hr. for incubating the confirmatory tubes. Absence of sufficient gas production in this time, they consider, disproves the original presumption. This seems only reasonable, since a coliform organism, able to produce adequate gas within 48 hr. when present perhaps in small numbers and possibly competing with other organisms in the primary tube, should well be able to produce a similar volume of gas within a similar time when large numbers are added in pure culture to the confirmatory tube. Unfortunately, however, the Ministry of Health Report (1939) offers no guidance on this point, and some workers have extended the period of incubation of the confirmatory test beyond reasonable limits. Bardsley (1934), for example, adopted a period of 10 days and accepted, as confirmation of a 48 hr. presumptive tube, cultures which require 5–10 days to produce gas. In this way she concluded that from 4297 primary tubes she obtained only one false presumptive reaction. By using the 48 hr. criterion recommended above, however, she would have recognized that her series contained a total of 460 false presumptive reactions, i.e. 10·7%. In our Memorandum (P.H.L.S. 1951) we have, therefore, suggested for water bacteriology a definition of coliform bacteria which restricts the term to organisms morphologically resembling *Bacterium coli* and capable of producing acid and gas in MacConkey broth within 48 hr. at 37° C. Adherence to this definition should ensure reasonable uniformity and sound procedure in the confirmatory test. In the work to be cited and described the limit of 48 hr. has not been exceeded.

FINDINGS OF PREVIOUS WORKERS

(*a*) *Thresh, Beale & Suckling*

Thresh *et al.* (1943) recorded and analysed the final results from 10,000 consecutive presumptive positive reactions obtained in the routine examination of water samples. The results of plating on to MacConkey agar were as follows:

Red (acid-producing) 'coli-like' colonies	9,520
Colourless (non-acid-producing) colonies and minute red colonies (most probably enterococci)	347
Complete absence of growth	133
Total	10,000

https://doi.org/10.1017/S0022172400015692 Published online by Cambridge University Press

Of the 9520 plates showing possible coliform colonies 9135 were proved by the confirmatory test to contain efficient gas producers. From the remaining 385 plates (i.e. 4·0 % of 9520) the confirmatory tubes failed to show adequate gas production within 48 hr. and the original presumption was regarded as disproved. In other words, the 'coliform' bacteria isolated could not be regarded as wholly responsible for the original fermentation. The false results from the 10,000 presumptive reactions thus totalled 865, as follows:

	No.	%
Absence of any aerobic colonies	133	1·33
Absence of any 'coli-like' colonies	347	3·47
'Coli-like' but non-gas-producing colonies	385	3·85
Total	865	8·65

In this series, therefore, 8·65 % of the presumptive reactions were considered false, or, in other words, the presumptive test proved to be 91·35 % accurate.

Where plating resulted in a complete absence of growth it was assumed that the original fermentation had been caused by anaerobes, such as *Clostridium welchii*, already reported by Greer (1928) as one of the commonest causes of false presumptive reactions in routine water analysis in the United States of America. Colonies colourless after 48 hr. appearing alone (or in association only with enterococci which are known to produce no gas from lactose) were presumed not to have been solely responsible for the original fermentation, since from their failure to produce acid from lactose in the solid medium it was considered unlikely that they would produce acid and gas in the same liquid medium (but see Table 2). Such colonies were, therefore, not tested and the cause of the original fermentation remained unexplained, being possibly either anaerobes or a mixture of organisms acting symbiotically. Where plating yielded only red 'coli-like' colonies which subsequently failed to produce gas, the original fermentation remained likewise unexplained but in both types of result it seemed fairly clear that coliform bacteria *per se* could not have been responsible for the gas in the presumptive tube.

(*b*) *Taylor*

The findings of Thresh *et al.* (1943) were supported by Taylor (1949) who added an analysis of results obtained in the routine examination of water samples in the laboratories of the Metropolitan Water Board (Mackenzie, 1938). Altogether 8843 presumptive positive tubes were plated out, and the resulting colonies were differentiated and identified. The false presumptive reactions amounted to 7·7 %, a percentage closely similar to that (8·65 %) reported by Thresh *et al.* (1943).

Taylor (1949) further classified his results according to the source of the sample, and demonstrated a progressive increase in the proportion of false presumptive reactions during the later stages of water purification. His results are summarized in Table 1.

It is not surprising that the total number of fermented tubes arising from Metropolitan Water Board chlorinated water is small (141), but it is noteworthy that such a large proportion of these were false reactions (32·6 %), of which nearly three-quarters were presumably caused by anaerobes. Anaerobes possibly constitute

a special problem for the Metropolitan Water Board since, like an increasing number of water authorities, it derives the bulk of its supplies from polluted river water which contains large numbers of clostridial spores. These spores may, to a considerable extent, be removed by filtration, but they can survive storage and a chlorination dosage sufficient to destroy vegetative bacteria. It seemed unlikely, therefore, that a countrywide survey would yield such high figures. Such a survey was commenced in 1949.

Table 1. *Results of differentiating* 8843 *presumptive reactions*

(Modified from Taylor (1949, p. 460))

				False presumptive reactions					
	Total presumptive reactions	True coliform organisms		Non-lactose-fermenters		Anaerobes		Total	
Source of sample	No.	No.	%	No.	%	No.	%	No.	%
River	2368	2336	98·6	20	0·8	12	0·5	32	1·4
Stored	1486	1383	93·1	79	5·3	24	1·6	103	6·9
Filtered	4848	4349	89·7	309	6·4	190	3·9	499	10·3
Chlorinated	141	95	67·4	14	9·9	32	22·7	46	32·6
Total	8843	8163	92·3	422	4·8	258	2·9	680	7·7

METHODS USED IN PRESENT INVESTIGATION

All presumptive positive tubes resulting from the examination of water samples in six laboratories—Birkenhead, Cambridge, Conway, Manchester, Newport (Mon.) and Oxford—were investigated particularly for positive rather than inferential evidence of the influence of anaerobes. All tubes were plated on to MacConkey agar for incubation at 37° C. for 48 hr. Those yielding red (acid-producing) 'coli-like' colonies after 24 hr. incubation were accepted as providing sufficient confirmation for the purpose of this investigation (cf. the 'colony-confirmed' presumptive test of the American *Standard Methods for the Examination of Water and Sewage*, American Public Health Association, 1946). Colonies colourless at 24 hr. but developing redness within 48 hr. were also similarly accepted as probable gas producers. Where all the colonies remained colourless at 48 hr. several were separately picked into MacConkey broth for incubation at 37° C. for 48 hr. A substantial proportion produced adequate gas within this period, and where this occurred the organisms were of course accepted as coliform bacteria responsible for the original fermentation.

The false presumptive reactions detected thus fell into two classes: (1) tubes which on plating yielded no colonies at all, (2) tubes which on plating yielded colonies colourless at 48 hr. and unable to produce gas in MacConkey broth. These cultures required special investigation for anaerobes, but it was impossible to delay this search until they could be clearly recognized. It has, for example, already been noted (Wilson & Miles, 1946) that *Cl. welchii* dies in a few days in media containing a fermentable carbohydrate, and we soon confirmed the need (already suggested by one of us, E. W. T.) for subculturing the presumptive positive tube

at the earliest possible moment. The following subcultures from the primary tube were, therefore, made whenever overnight incubation of the plates failed to yield indisputably red colonies (i.e. with plates yielding no growth or only colourless colonies):

(*a*) A loopful was transferred to a fresh MacConkey broth tube for incubation at 37° C. for 48 hr. in order to test that the original fermentation could be reproduced.

(*b*) A loopful was plated on to blood agar for anaerobic incubation at 37° C. for 24 hr.

(*c*) 1–2 ml. were transferred to a litmus milk tube for incubation at 37° C. for 5 days.

(*d*) From the deposit in the fermented tube a small amount was removed with a sterile Pasteur pipette for examination by Gram's stain.

Failure to reproduce the original fermentation in (*a*) would indicate that the organisms responsible were no longer alive. In such sterile subcultures, the presence in (*d*) of large Gram-positive bacilli at least suggested that anaerobes had been present and might be considered responsible for the original fermentation. It was usual to find large numbers of these bacilli in the deposit in those tubes which on subculture produced a sterile plate. *Cl. welchii*, if present and viable in the tube, however, should produce its characteristic colonies on blood agar and a typical 'stormy clot' in litmus milk.

RESULTS

Over 10,000 tubes were plated and investigated as described. In Table 2 the results, classed according to whether the sample was from chlorinated or non-chlorinated water, are shown for each laboratory.

Of the total 10,436 tubes plated, 10,310 (98·8 %) yielded colonies which were either red and 'coli-like' or capable of producing acid and gas in MacConkey broth within 48 hr. Tubes yielding colourless colonies incapable of gas-production or no colonies amounted to 126 (1·2 %). Only 46 of these arose from the 8975 fermented tubes derived from non-chlorinated water, whereas 80 resulted from the 1461 tubes derived from chlorinated water. The percentages of these false presumptive positives in non-chlorinated and chlorinated water were thus respectively 0·5 and 5·5 (Table 2). From the former series *Cl. welchii* was grown only twice, but from the latter 41 times out of 80. In the few tests where plating yielded no colonies at all and *Cl. welchii* could not be isolated it was usually possible to demonstrate microscopically the presence of numerous large Gram-positive bacilli in the fermented tube.

When the results from the various laboratories are compared it is evident that considerable differences exist. Birkenhead and Cambridge obtained no presumptive positive reactions from chlorinated water and relatively few, with no obviously false, presumptive positives from non-chlorinated water. Oxford reported hardly any. Conway, Manchester and Newport reported most false presumptive positives. At Newport, and to some extent at Oxford, the sources from which water, which is subsequently chlorinated, are obtained are subject to considerable sewage

Table 2. *Analysis of presumptive positive reactions grouped according to area and nature of sample*

Nature of sample	Area	Total *fermented* tubes	Confirmed presumptive positive tubes: Red colonies	Confirmed presumptive positive tubes: Colourless colonies becoming red or *producing acid and gas*	False presumptive positive: Colourless colonies *not producing* acid and gas	False presumptive positive: Sterile plates	*Cl. welchii* isolated from false presumptive positive tubes
Non-chlorinated	Birkenhead	450	450	0	0	0	0
	Cambridge	488	488	0	0	0	0
	Conway	1073	950	109	13	1	0
	Manchester	3017	3002	0	15	0	0
	Newport, Mon.	1711	1494	204	13	0	1
	Oxford	2236	2232	0	4	0	1
	Total	8975	8616 (96·0 %)	313 (3·5 %)	45 (0·5 %)	1 (0·0 %)	2
		100 %	8929 (99·5 %)		46 (0·5 %)		
Chlorinated	Birkenhead	0	0	0	0	0	0
	Cambridge	0	0	0	0	0	0
	Conway	154	138	2	10	4	2
	Manchester	905	882	0	7	16	7
	Newport, Mon.	274	169	64	9	32	31
	Oxford	128	126	0	1	1	1
	Total	1461	1315 (90·0 %)	66 (4·5 %)	27 (1·9 %)	53 (3·6 %)	41
		100 %	1381 (94·5 %)		80 (5·5 %)		
All sources	Grand total	10,436 (100 %)	9931 (95·2 %)	379 (3·6 %)	72 (0·7 %)	54 (0·5 %)	43
			10,310 (98·8 %)		126 (1·2 %)		

pollution, but this does not apply at Conway and Manchester where a considerable number of false presumptive reactions were also reported. The sources from which the Birkenhead and Cambridge waters were obtained were not liable to pollution by sewage.

The influence of anaerobes on the presumptive coliform count in samples of chlorinated water is illustrated by a series collected at Newport. Of 1035 samples of chlorinated water examined 206 gave positive results by the presumptive coliform test. In 33 of these 206 samples the fermentation was due entirely to anaerobes and in ten more it was due in part to anaerobes.

The error caused by anaerobes in an individual sample of chlorinated water may be very considerable. In one of the samples examined at Newport, for example, presumptive coliform and faecal coli counts of 25 per 100 ml. were entirely due to anaerobes, as these fermented the lactose in MacConkey broth both at the initial incubation temperature of 37° C. and at the confirmatory incubation temperature of 44° C. (see p. 276).

DISCUSSION

The present investigation was aimed not at assessing the absolute accuracy of the presumptive coliform test, but rather at showing that anaerobic spore-bearing organisms—and in particular *Cl. welchii*—might be an important cause of false presumptive positives in certain types of water, particularly in chlorinated water.

From Table 2 the percentage of false presumptive positives associated with colourless colonies which fail to produce acid and gas from lactose or with a failure of colonies to develop on plating is seen to be 1·2. In the present series the red colonies were not examined for gas production. These numbered at least 9931, as some of the 379 colonies classified in Table 2 as 'colourless becoming red or producing acid and gas' developed redness at 48 hr. but were not further tested. In two investigations in which red colonies have been tested for gas production the percentage of red colonies failing to produce gas in 48 hr. has been shown to be 4·0 (Thresh *et al.* 1943) and 4·8 (Taylor, 1949). On this basis it can be estimated that the total false presumptive positives in our series would have been between 5·2 and 6·0 % which is somewhat lower than the figure of 8·65 % obtained by Thresh *et al.* and that of 7·7 % obtained by Taylor.

The extra accuracy obtainable by full confirmation involves too much labour to be practicable for daily routine work in many laboratories where the emphasis now rightly lies on simple tests frequently repeated. Even colony confirmation places a severe strain on a routine laboratory. Our series indicates that, with non-chlorinated waters, the difference in accuracy between the presumptive test and partial confirmation is only 0·5 %. It would seem, therefore, that with unchlorinated supplies the presumptive test gives an accurate enough indication of the coliform content.

This conclusion is not valid for chlorinated supplies where partial confirmation alone reveals an error of 5·5 %. Most samples of chlorinated water yield completely negative presumptive results. In the few instances where presumptive positive

tubes are obtained it would involve relatively little extra trouble to continue the investigation to the stage of plating and even further if necessary. The appearance on the plate of red colonies confirms with reasonable accuracy the presence of coliform bacteria. The appearance even of colourless coli-like colonies indicates the presence in the water of vegetative bacteria which ought to have been killed by effective chlorination. Without testing for gas production such colonies may, therefore, be regarded as an indication of insufficient purification of the water supply and may be classed as coliform bacteria. Often, however, the plate shows no growth. It is the contention of the writers that such sterile plates are an indication that the original fermentation was probably caused by anaerobes.

If the subcultivation has been performed with good technique the production of a sterile aerobic plate admits of only two explanations:

(1) The original fermentation was caused by bacteria which died in the fermented tube before subculture was performed.

(2) The original fermentation was caused by anaerobes such as *Cl. welchii* which, even if still alive, would not grow on the plate.

The former explanation is not impossible, but experimental support for it has not been obtained. When fermented tubes have been examined for survival of coliform organisms, they have often been isolated on each of seven or more successive days even when, as sometimes happened, the tubes were retained in the 37° C. incubator. There may, nevertheless, be some coliform strains which are unduly sensitive to the acidity produced, but even these should be obtained alive if the fermented tube is subcultured within a few hours of the appearance of adequate gas.

Believing the second explanation to be the true one, we endeavoured, when plating yielded no coliform bacteria, to demonstrate the presence of anaerobes. In 41 out of the 80 obviously false presumptive positives obtained from chlorinated water we obtained incontrovertible evidence of the presence of *Cl. welchii* either by demonstrating the characteristic colonies anaerobically on blood agar or by producing the typical stormy clot in milk. In some of the remaining tests microscopic examination of the deposit in the fermented tube demonstrated the presence of numerous large bacilli morphologically resembling clostridia, suggesting that the organisms which had died were not coliform bacteria but more probably anaerobic bacilli. That we should have been able to recover the anaerobes alive from only half the false presumptive positive tubes is not remarkable when it is considered that the fermented tube, besides its period of 24 or 48 hr. in the incubator, had stood on the bench overnight while awaiting the result of plating. To obtain a higher rate of recovery it is necessary to subculture for anaerobes as soon as the fermentation is produced, i.e. at the same time as the plating is performed. Taylor (personal communication) adopted this procedure for a period on all fermented tubes arising from chlorinated water and was able to cultivate *Cl. welchii* from 150 (95 %) out of 158 tubes which, when subcultured on MacConkey plates, gave no growth. That a presumptive positive reaction is false and is caused by anaerobes can often but not always be forecast by the poor acidity and large

volume of gas produced. Immediate subculture of these tubes for anaerobes is usually successful.

We have shown (Table 2) that in 41 (77·4 %) out of the 53 tests in which the aerobic plate was sterile *Cl. welchii* was present in the original presumptive positive tube. The production of a sterile aerobic plate is therefore a reasonable guide to the presence of anaerobes which are thus shown to account for a large proportion of the false presumptive positives obtained from chlorinated water.

A chlorinated water is rightly expected to show no coliform bacteria in 100 ml. Such a water may be free from coliform bacteria and yet give a false presumptive coliform count due to anaerobes of as many as 25 per 100 ml. Moreover, anaerobes can also interfere with the faecal coli count since, if they remain alive, they can produce gas in MacConkey broth incubated at 44° C. This particular error can be largely avoided by the use of the brilliant green bile lactose broth developed by Mackenzie, Taylor & Gilbert (1948).

The presence of coliform bacteria in chlorinated water suggests inadequate treatment or subsequent deterioration and indicates the possibility of danger. The presence of anaerobes without coliform bacteria in chlorinated water indicates no similar likelihood of danger, although it may reflect adversely on the filtration process. Academic considerations apart, therefore, it is unfair to regard as contaminated by coliform bacteria a chlorinated water which is contaminated only by anaerobes. Whereas, therefore, the presumptive coliform test is sufficiently accurate for the assessment of unchlorinated supplies, chlorinated waters which react positively to the presumptive test should not be assessed until the results are checked at least by plating on MacConkey agar.

SUMMARY

The investigation of 10,436 fermented tubes arising in the presumptive coliform examination of samples of water at six laboratories in England and Wales has shown that, with unchlorinated supplies, the unconfirmed presumptive test gives sufficiently accurate results. Full confirmation of all presumptive positive tubes of these waters is impracticable as a routine and 'colony-confirmation' gives a correction of only 0·5 %. With chlorinated waters, however, 'colony-confirmation' discloses an error of 5·5 %, largely due to the presence of anaerobes. This error can also affect the faecal coli (44° C.) count and may be so large in individual samples that assessment of chlorinated supplies should not be based on the presumptive test until this has been checked at least by plating.

REFERENCES

AMERICAN PUBLIC HEALTH ASSOCIATION (1946). *Standard Methods for the Examination of Water and Sewage*, 9th ed. New York: American Public Health Association.

BARDSLEY, D. A. (1934). *J. Hyg., Camb.*, **34**, 38.

GREER, F. E. (1928). *J. infect. Dis.* **42**, 501.

MACKENZIE, E. F. W. (1938). *33rd Annual Report Director of Water Examination, Metropolitan Water Board.*

MACKENZIE, E. F. W., TAYLOR, E. W. & GILBERT, W. E. (1948). *J. gen. Microbiol.* **2**, 197.

MINISTRY OF HEALTH (1939). The bacteriological examination of water supplies. *Rep. Publ. Hlth. med. Subj., Lond.*, no. 71.

PUBLIC HEALTH LABORATORY SERVICE (1951). The bacteriological control of drinking water. (Memorandum prepared by the P.H.L.S. Water Sub-Committee.) *Mon. Bull. Minist. Hlth Lab. Serv.* **10**, 18.

TAYLOR, E. W. (1949). *The Examination of Waters and Water Supplies*, 6th ed. (Thresh, Beale and Suckling). London: Churchill.

THRESH, J. C., BEALE, J. F. & SUCKLING, E. V. (1943). *The Examination of Waters and Water Supplies*, 5th ed. London: Churchill.

WILSON, G. S. & MILES, A. A. (1946). *Principles of Bacteriology and Immunity*, 3rd ed. p. 887. London: Edward Arnold and Co.

(*MS. received for publication* 15. VII. 52.)