THE EIJKMAN TEST FOR FAECAL COLI IN THE BACTERIOLOGICAL EXAMINATION OF WATER SUPPLIES[1]

A SURVEY AND DISCUSSION OF THE EXPERIMENTAL WORK FROM 1929 TO THE PRESENT DAY WITH A STUDY OF 104 WATER SAMPLES AND OF 602 CULTURES

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[1] This paper formed part of a thesis submitted to the University of Cambridge for the degree of M.D.

Introduction

It is now nearly forty years since Eijkman (1904) propounded his thesis that *Bact. coli* from the intestine of warm-blooded animals was capable of fermenting glucose with the production of acid and gas at a temperature of 46° C., whereas *Bact. coli* isolated from the intestine of cold-blooded animals was not. It is remarkable, therefore, that only during the last few years has the test begun seriously to be considered as a valuable routine measure in the bacteriological examination of water supplies.

The reason for this is not far to seek. For about thirty years the test was tossed from one investigator to another to be alternately praised and blamed, and the various workers contented themselves with agreeing or disagreeing with Eijkman's original findings without any real inquiry into the *reasons* underlying their contradictory results. Besides, during this period the volume of investigation was not great, and it has been during the last ten years that the crucial factors in technique have been brought to light by a large number of writers.

Until the publication of Wilson's report on the Bacteriological Grading of Milk in 1935, interest in this test had been centred chiefly abroad, notably in America, but now that more work is being undertaken in this country the time seems opportune for a review of the literature of the subject and for a discussion of the opinions held to-day as to the value of the test in water examination.

Part I. Historical survey

The Eijkman test

It is proposed to pass rapidly over the work of the early continental investigators to reach the period of more fruitful investigation which began in about the year 1929.

Konrich (1910) and Hehewerth (1911) both found that a considerable number of strains of undoubted faecal *Bact. coli* failed to develop in glucose broth at 46° C., and with this criticism of his work Eijkman (1912) himself substantially agreed, saying that a negative result could not be regarded as proof of the absence of *Bact. coli*.

Flu (1915) considered the test a valuable indication of recent contamination, while de Graaff (1922) found that incubation at 46° C. inhibited the *aerogenes* organisms.

Brewster (1929), using glucose broth at 46° C., found that nearly all the lactose-fermenters produced acid and gas. He considered that the explanation of the failure of the method to give satisfactory results in the tropics was that the soil bacteria had adapted themselves to temperatures at which the soil bacteria of temperate climates refuse to develop.

Wilson (1929) incubated MacConkey plates at 45° C. and found that a positive result invariably indicated the presence of faecal *Bact. coli* but that a negative result had not the opposite significance.

Perry (1929), in an investigation of oysters, came to the conclusion that while the faecal *Bact. coli* was certainly 'Eijkman-positive' a great many methyl-red-negative coliforms behaved in the same way; he nevertheless recommended the use of the test with subsequent plating and confirmation.

Leiter (1929) made a very careful study of the value of Eijkman's test on samples of water and of human faeces; he also inquired into the truth of Eijkman's assertion that the test was able to distinguish between *Bact. coli* obtained from warm- and from cold-blooded animals. Employing, with incubation at 46° C., a medium very similar in composition to Eijkman's original medium, he inoculated water samples into it, and performed at the same time parallel inoculations into the lactose broth of American 'Standard Methods' at 37° C.

The results showed that the Eijkman test was usually complete within 24 hr. and that it was almost perfectly selective for indole-producing *Bact. coli*; 'Standard Methods' revealed a much smaller number.

Leiter encountered 10% of samples in which *Bact. coli* was detected by the Eijkman test but in which 'Standard Methods' showed only the *aerogenes-cloacae* group. He concluded that these organisms had overgrown *Bact. coli* at 37° C. but had been inhibited or destroyed at 46° C. This worker's investigations on human faeces showed the specificity of Eijkman's test in an equally favourable light, demonstrating in addition a practically perfect negative correlation between dextrose fermentation at 46° C. and citrate-utilization.

Turning his attention to Eijkman's claims regarding the possibility of distinguishing between *Bact. coli* from the faeces of warm- and of cold-blooded animals, Leiter found that 100% of the strains of *Bact. coli* which he isolated from the intestinal contents of chicken, pigeon, pig, horse and cow were 'Eijkman-positive' and nearly all were indole-positive. In the case of cold-blooded animals—fish and frogs—the results were not so clear in that about one-third of the strains of *Bact. coli* isolated were Eijkman-positive and that the correlation between the Eijkman, indole and citrate tests was poor.

The above results were obtained when using material from animals killed in the spring; a further series killed in the autumn produced an even greater number—about 50%—of Eijkman-positive strains. Leiter concluded from these results that the autumn series were probably actively feeding and that they may have derived some strains from the environment via the faeces of warm-blooded animals; he thought it evident that the presence of cold-blooded animals in a water supply might cause a positive result in the 37° C. test.

Eijkman's original findings, therefore, were in the main corroborated by Leiter. He supported the use of the 46° C. temperature saying that the *aerogenes-cloacae* group grew just as well as *Bact. coli* at temperatures below 45° C. and found that even at 49° C. 50% of strains of the latter gave good gas production. He agreed with Eijkman that *Bact. coli* could be isolated in almost pure culture by this test.

Leiter's final conclusions were that the test was selective for *Bact. coli* from water, that it confirmed the presence of *Bact. coli* in a higher percentage of samples than did 'Standard Methods', and that it would pass as satisfactory waters far removed from the possibility of contamination by warm-blooded animals when those same waters would have been condemned by 'Standard Methods'.

Brown & Skinner (1930) studied human faeces and found the test highly specific for *Bact. coli* though they disagreed with Leiter in that they found 48 hr. incubation necessary for some cultures. They applied the test to water samples and considered that it failed to detect all the faecal *Bact. coli* and that a fair number of *aerogenes* and atypical coliform strains were involved in a positive Eijkman test. They noted that positive tubes after 48 hr. contained few or no viable organisms, an important observation that was to bear fruit later.

Ruchhoft, Kallas, Chinn & Coulter (1931) also found that some *aerogenes* strains grew well at 46° C. and considered that a positive result on water samples at this temperature did not indicate the presence of faecal *coli* from warm-blooded animals alone.

Wagner (1931) questioned the specificity of the test for *Bact. coli* and found it very unreliable; he gave hardly any details of his technique, and the extreme variability in the results which he obtained suggests that some part of it was insufficiently standardized.

Taylor & Goyle (1931), working in the tropics, confirmed the specificity of the test for *Bact. coli* from warm- as against that from cold-blooded animals and, as a result, had to postulate the survival of faecal *Bact. coli* in soil to justify the positive Eijkman results they obtained from water samples which came from areas considered to be free from obvious human and animal pollution. They then showed that faecal *coli* had in fact a considerable survival period in soil at a temperature of 80° F. They used the test on water samples and found that the results tallied to a remarkable degree with the probable sanitary quality of the water and were of far more value than those obtained by the usual incubation at 37° C. followed by isolation and differential tests.

Burke-Gaffney (1932), also working in the tropics, compared the results of incubation of water samples in lactose-bile-salt broth at 37 and 46° C. He found the test very unreliable, as more organisms of the *aerogenes* than of the coli type developed at 46° C. In this finding he was in agreement with the work of Brewster (1929) and of Brown & Skinner (1930), but it is difficult to understand such results in the light of more recent experience since even a temperature of 44° C. is nowadays found to inhibit *aerogenes* strains almost completely. Brewster's suggestion that the latter organisms have, in the tropics, adapted themselves to higher temperatures is possibly the explanation.

Kingsbury (1932), another tropical worker, also reported very favourably on the test both as to specificity for *Bact. coli* and as a practical indicator of the sanitary quality of waters.

One of the first real steps forward in setting the test on a sound basis was now taken by Williams, Weaver & Scherago (1933), who, prompted by the observations of Brown & Skinner (1930) that many positive Eijkman tubes failed to confirm, set out to prove that the organisms were killed by the high concentration of acid produced in the medium.

To this end they determined the relative amounts of acid produced in the original Eijkman medium (containing 1·4% glucose) and in their own 'modified Eijkman medium' (containing 0·5% glucose) and found that 40% less acid was produced in the latter.

In comparative experiments on known coliform strains from water and faeces, using their 'modified Eijkman medium' at 46° C., as against 'Standard Methods' lactose broth at 37° C., they found the Eijkman test highly specific for faecal *Bact. coli*, none of the *aerogenes* strains producing gas at the higher temperature.

These workers also performed comparative tests on routine water samples using modified Eijkman medium, original Eijkman medium and 'Standard Methods' lactose broth at 37° C. No 'modified Eijkman' tube which was negative after 24 hr. ever became positive after 48 hr. They also found that the modified Eijkman test eliminated a large number of 'Standard Methods' positives, which had failed to confirm. The modified Eijkman test in several cases detected the presence of faecal *Bact. coli* when the water had been passed by 'Standard Methods', and never failed to show *Bact. coli* when it was present.

Williams and his colleagues made a further suggestion to account for the variable results of the Eijkman test, namely, that the irregularities in the growth of pure strains of *Bact. coli* experienced by previous investigators may have been due to over-cultivation of the organisms on artificial media. They found that changes in fermentation characteristics occurred after several transfers, the property of gas formation being to some extent lost.

These authors sum up by saying that since, in their opinion, *Bact. aerogenes* does not indicate pollution, the use of the modified Eijkman procedure prevents the condemnation of many potable waters and that the test is more reliable than 'Standard Methods' for the detection of faecal coli.

At about the same time, Perry & Hajna (1933) also suspected excessive acid formation in the medium of causing some strains of *Bact. coli* to fail to grow at 46° C. and therefore produced a medium containing only 0·3% of glucose and a phosphate buffer. With this medium, every strain of *Bact. coli* promptly produced gas at 46° C. and every culture was viable even after 96 hr., whereas growth on plates was never obtained after 48 hr. from the original Eijkman broth. The final *p*H in their medium was 5·6 as against 4·5 in the original Eijkman medium.

Using a carefully regulated incubator, these workers showed that the test with their new medium was highly specific for *Bact. coli* and that it detected more *Bact. coli* in faeces than did standard lactose at 37° C. In fine, they

concluded that Eijkman's fundamental observations were correct when using a suitable medium and carefully controlled temperature, but that his original medium contained too much glucose, thereby causing the death of the culture by reason of the high acidity produced.

Skinner & Brown (1934) drew attention to the contradictory nature of the literature and suggested that differences in technique must be responsible for it. They remarked that the tests had sometimes been done in small tubes, sometimes in large, sometimes in incubators, sometimes in water-baths, and concluded that the use of small tubes and water-bath incubation were the only means of securing a controlled and uniform temperature in the medium. Lack of uniformity in these details might, they thought, explain the divergent results.

They made parallel inoculations of faecal suspensions into three media: (1) Eijkman glucose broth, (2) Bulir's mannitol broth, (3) lactose broth, the first two being incubated at 46° C. and the third at 37·5° C.

In the case of the glucose and mannitol media, duplicate sets were inoculated, one set being placed at once in a water-bath at 46° C., the other into a water-bath brought slowly up to 46° C. from cold. The water-baths had special regulators and the thermometers were certificated. The lactose tubes were placed in an incubator.

It should be mentioned here that Bulir's (1907) test is a modification of the Eijkman test and consists in incubating at 46° C. a peptone-beef-infusion broth containing 0·66 % of mannitol. Some continental workers have reported on it as being superior to the Eijkman test.

These workers found that the lactose broth showed more *Bact. coli* than either of the other methods, and concluded that neither the Eijkman nor Bulir tests could be depended upon to detect anything like all the *Bact. coli* of faecal origin. Both tests were found particularly to miss some of the faecal *coli* if the temperature of 46° C. was reached immediately after inoculation.

In this connexion, these authors say: 'It is evident that many cells which would fail to produce gas at 46° C. can do so if gradually brought to that temperature in culture media. This is particularly true in the Eijkman test. Possibly this is a partial explanation of the divergent results of various investigators.'

Skinner & Brown also reported, that in their opinion, the Eijkman test did not differentiate reliably between *Bact. coli* from warm- and from cold-blooded animals, and that 48 hr. incubation was necessary for the test.

Webster & Raghavachari (1934), testing waters in the Madras Presidency by MacConkey's broth at 37° C. and Eijkman's medium at 46° C., found that quite frequently samples showing the presence of true *Bact. coli* in the 37° C. tubes, failed to ferment the glucose at 46° C. They concluded that the Eijkman test sometimes failed to reveal pollution, that it was insufficiently sensitive and added no useful information to the results of the usual routine tests.

Hitherto the question of the optimum temperature for the test had not

been given the attention it deserved, but with the paper of Levine, Epstein & Vaughn (1934) it came at last under effective review.

They performed parallel incubation with a temperature *in the medium* of, in one case 43–44° C., and in the other of 45·5–46° C., and found that all *Escherichia* strains grew well in Eijkman's broth and standard lactose broth at the lower temperature, but that they were markedly inhibited as regards gas production at 45–46° C. The *Aerobacter* strains were strongly inhibited at the lower temperature.

The temperature theme was now conclusively developed by Wilson *et al.* (1935) in their exhaustive research into the bacteriology of milk. This work is of such interest and importance that their remarks are worth quoting in some detail.

These workers used the modified Eijkman medium of Williams *et al.* (1933) and showed that a temperature of 46° C. was too high, since growth of *Bact. coli*, much less gas production, could not be relied upon. At 44° C., however, practically all indole-positive strains of *Bact. coli* grew well and produced gas. No strains of *aerogenes* produced gas at this temperature and very few even grew at all. A temperature of 44° C. *in the medium*, not *in the incubator*, was employed.

They recommended that if an *incubator* was used it should generally be kept at 45° C. because the temperature within was never quite uniform and the medium probably never reached the temperature of the surrounding air. They stressed the need for using a water-bath at 44° C. in preference to an incubator.

Taking gas production within 48 hr. as a positive result, it was found that all intermediates and all but one *aerogenes* strain were Eijkman-negative, while a very high percentage of strains of *coli* were positive. The correlation between the Eijkman and indole tests was striking, as was the negative correlation between the Eijkman test and growth in citrate.

These workers ascribed the conflicting views on the Eijkman test to variations in technique and they particularly suggested the non-standardization of incubator temperatures as the most probable explanation. They thought it likely that those workers who reported favourably on the test had used incubators giving a constant temperature of 44° C. in the tubes, while those who reported unfavourably had been working with incubators whose temperatures were either too high, too low or inconstant.

Wilson and his co-workers observed that 42° C. was too low, as it permitted occasional *aerogenes* strains to form gas, whilst 46° C. was too high, and inhibited many of the *Bact. coli*.

They observed too that gas production was better in MacConkey broth at 44° C. than in the modified Eijkman medium of Williams.

The conclusions drawn by these investigators were that the Eijkman test, if performed according to their recommendations but using MacConkey broth, was a test of great value; that no other test alone was so rapid or so well able

to pick out strains of faecal *coli* and that correct temperature was of vital importance in its performance.

They suggested a classification of the coliform group based on the Eijkman test, and their classification is given in full in Table 1 as reference will constantly be made to it.

It will be noted that gelatin liquefaction serves only to distinguish between *Bact. aerogenes* and *Bact. cloacae*, a distinction which is without object in water bacteriology since the sanitary significance of these two organisms is practically the same.

The ability to form gas in MacConkey broth at 44° C. is seen to be characteristic of only two important types, *Bact. coli* type I and Irregular type II. The second is rare enough to be of little significance, so that at any rate in this country, it may for practical purposes be said that gas production at 44° C. is specific for *Bact. coli* type I.

Table 1. *Classification of coliform strains*

Type	M.R.	V.P.	Citrate	Indole	Eijkman 44° C.	Gelatin liquefaction
Bact. coli, type I	+	−	−	+	+	−
Bact. coli, type II	+	−	−	−	−	−
Intermediate, type I	+	−	+	−	−	−
Intermediate, type II	+	−	+	+	−	−
Bact. aerogenes, type I	−	+	+	−	−	−
Bact. aerogenes, type II	−	+	+	+	−	−
Bact. cloacae	−	+	+	−	−	+
Irregular I, *coli*-like 1	+	−	−	+	−	−
Irregular II, *coli*-like 2	+	−	−	−	+	−
Irregular III, *coli*-like 3	+	−	−	−	−	+
Irregular IV, intermediate-like	+	−	+	−	−	+
Irregular V, *aerogenes*-like 1	−	+	−	−	−	−
Irregular VI, *aerogenes*-like 2	−	+	+	−	+	−
Irregular VII	−	−	+	+	−	−
Irregular VIII	−	−	−	−	−	−

They also developed three methods for the practical use of the 44° C. test in routine water or milk examination: their method II consists in transferring a series of dilutions of the water or milk to a duplicate set of Petri plates; melted MacConkey agar is then poured into each plate; one set of plates is incubated at 37° C., the other at 44° C. and the red colonies are counted after 2 days. The 37° C. plate count gives the total coliform count, the 44° C. plate count gives the number of faecal *coli* only.

The authors point out that before this method can be considered valid it is necessary to assume that all colonies growing at 44° C. are of faecal *coli* type, while those growing at 37° C. may include all the coliform types. The first assumption, they say, is probably justified, the second is vitiated by the presence of other organisms which give rise to red colonies but which are not coliform organisms producing acid and gas. They conclude that though the method gives a rapid and fairly accurate estimate of the faecal *coli* count, it tends to over-estimate the total number of coliforms.

To this objection which the authors bring forward there may be added one which would tend to have the opposite effect upon the results; namely, that since many recent workers believe in the existence of genuine slow- and non-lactose-fermenting coliforms, the total coliform count might be too low by the number of coliforms which failed to produce red colonies.

Wilson's method III consists in making parallel inoculations of a water sample into two sets of tubes of MacConkey broth, one set being incubated at 37° C. and the other at 44° C. Each positive tube of the 37° C. set is then subcultured into Koser's citrate medium.

The validity of this method rests on the assumption that only faecal *coli* can form gas in MacConkey broth at 44° C., whereas organisms forming gas at 37° C. may be either *Bact. coli* I or II, *aerogenes-cloacae* or intermediate types. If they prove to be citrate utilizers they may be of the *aerogenes*, *cloacae* or intermediate types; if they fail to grow in citrate they are of *coli* I or II type.

By means of probability tables the faecal *coli* count is estimated from the number of tubes positive at 44° C., the total coliform count from those positive at 37° C. and the intermediate-*aerogenes-cloacae* count from the positive citrate tubes.

The authors are of opinion that this method is very valuable as a rapid indicator of the number of coliform organisms of all types but observe that difficulties arise with it if probable numbers are ascertained by probability tables because it may happen that the faecal *coli* count appears as higher than the total coliform count, which is absurd; the coliform count should, they say, be taken on either the 37 or 44° C. figure, whichever is the higher. They do not consider this method a good basis on which to calculate the *coli-aerogenes* ratio.

Method IV consists in the inoculation of the water sample into MacConkey broth tubes with incubation at 37° C. for 48 hr. followed by subculture of each positive tube into both citrate and MacConkey broth which is incubated at 44° C. All three counts are again read off from probability tables.

This method takes slightly longer than method III, but is recommended if all three counts are desired and is of particular value for determining the *coli-aerogenes* ratio.

Perry & Hajna (1935) experimented with their modified Eijkman medium (Perry & Hajna, 1933) in connexion with the examination of oysters, crabmeat and other substances.

Parallel tests were performed with their modified medium as against standard lactose broth with results which showed that the modified Eijkman medium was more selective and efficient for the isolation of *Bact. coli*.

They pointed out that, using the Eijkman test, much less work was involved because on plates made from Eijkman tubes *Bact. coli* was often the only organism present, whereas on plates made from lactose tubes, several members of the group were usually present. In their work, a temperature of 46° C. *in the tubes* was insisted upon, and an incubator was used. They found that

more *Bact. coli* were recovered than when the *incubator* temperature was 46° C. This finding is difficult to understand in the light of more recent work, since it is now well established that even an *incubator* temperature of 46° C. is too high.

Webster (1935) applied the test to Madras waters and confirmed the work of Webster & Raghavachari (1934) by finding that only twenty-three out of forty strains of true *Bact. coli* produced gas within 48 hr. in Eijkman's medium at 46° C.

This worker decided to try MacConkey's broth at 46° C. and also Williams's modified Eijkman medium (Williams *et al.* 1933). To do this, parallel tests on water were made using the above two media at 46° C. and MacConkey broth at 37° C. The results showed a great superiority for the 37° C. series, for Williams's medium inhibited many of the *Bact. coli*. The results with MacConkey broth at 46° C. were very irregular, and though *aerogenes* and intermediate strains were almost completely inhibited, many strains of true *coli* were also not revealed. It certainly appears as if the excessively high temperatures were responsible for the inhibition of true *coli* found by Webster.

Harold (1935), at the Metropolitan Water Board, made some experiments with a temperature in the tubes of 44·5° C., every tube being brought up to temperature before inoculation. Duplicate sets for each water sample were inoculated and incubated at 42 and 45° C. respectively. MacConkey broth was used in each case and readings were made at 18 hr. Isolation of *Bact. coli* was most frequent at the lower temperature, so some experiments were done which showed that, to give a positive result at 44·5° C., the original inoculum must contain more organisms than were needed at 37° C. Harold concluded that a large percentage of typicals did not survive at 44·5° C.

Hajna & Perry (1935) compared several special media incubated at 37° C., with their modified Eijkman medium (1933) incubated at 46° C., in the examination of samples of raw sewage. They obtained more positive tubes in the Eijkman series than in the lactose broth series, and of the former, 95·8% confirmed as *Bact. coli*, in comparison with only 87·8% of the latter. They concluded that the Eijkman method was superior to all other methods for the isolation of *Bact. coli*.

Topley & Wilson (1936) summed up the position at the time by advocating exact standardization of the temperature of incubation to 43–45° C. in the medium. They advised the use of a water-bath rather than an incubator, considered that the value of the test was greatly enhanced by the replacement in the medium of glucose by lactose, and that MacConkey's broth was the best medium.

They concluded that the test was better than any other single test for picking out typical faecal coli.

Harold (1936) continued, during the whole of 1936, the experiment at 42 and 45° C. previously mentioned. He formed the opinion that if the higher temperature was substituted in routine work, fewer unsatisfactory samples

would be picked out and that over-estimation of purity would supervene, particularly in the case of filtered waters.

One of the most interesting and informative papers in the literature is that by Minkevich, Alexandrov & Soboleva (1936) who used Bulir's mannitol broth with incubation at 46° C. in an investigation of *Bact. coli* from human faeces and found that a high percentage of typical strains produced gas. They considered that coliform bacilli from the intestines of cold-blooded animals were incapable of fermentation at this temperature.

Experiments were made to test the survival of faecal *Bact. coli* at 37 and at 46° C. by means of duplicate inoculations of river water into media at these two temperatures, the 46° C. tubes being heated to that temperature immediately after inoculation. The results showed that the higher temperature inhibited the development of *Bact. coli*, and this the authors explained by saying that in an incubator adjusted to 46° C. the temperature falls by several degrees after loading and does not rise to 46° C. again for several hours, so that at first development of *Bact. coli* takes place at a lower temperature. They even went so far as to try 4 hr. incubation at 37° C. with transfer to 46° C. at the end of that period, holding that the procedure gave better results, as the growths were by then strong enough to support the higher temperature.

Experiments were performed on the lines of those first mentioned except that the 46° C. tubes were allowed to take about 5 hr. after inoculation to reach their proper temperature, and it was then found that the number of tubes fermented at 37 and at 46° C. showed but little discrepancy.

The next experiment was to find 'the temperature which would not under any conditions prevent the development of even single cells of *B. coli communis* in fermentation tests in Bulir's medium'. This temperature was found to be 43·5° C.,—even 44° C. causing some inhibition—and the full advantage of the 46° C. temperature, that is, the elimination of non-faecal members of the *coli-aerogenes* group, was retained.

Minkevich and his co-workers then determined the relative quantities of gas produced by *B. coli communis* at 37 and at 43·5° C., as well as the influence of temperature on the rate of reproduction of this organism.

They found that the maximum amount of gas was produced at 43·5° C, in the first 24 hr., hardly increasing at all during the second 24 hr. At 37° C. there was a delay, and only after the lapse of 48 hr. did gas production rise to equal that at 43·5° C.

Paradoxically, the figures for reproduction rate were found to be in inverse proportion to those for gas production, and further experiment threw light on these seemingly antagonistic facts. Maximum gas production at 43·5° C. was shown nearly to be reached after 16 hr. of incubation, and it was also discovered that after reproduction had attained its peak rate it was replaced by a phase of progressive dying of the organisms because of the accumulation of acids.

The 'dying phase' took place much more rapidly at 43·5 than at 37° C., which explains why the medium was often sterile after 48 hr. incubation.

This work is very illuminating and corroborates the view of Wilson *et al.* (1935) as to the correct temperature for incubation; the authors consider that there is a danger of obtaining false positives if incubation is prolonged beyond 24 hr., but admit that some strains of *B. coli communis* may need a longer period to give a positive result.

Minkevich, Joffe & Shafir (1936) found that Bulir's medium at 43° C. was more selective for faecal *Bact. coli* than 'Standard Methods' lactose broth at either 37 or 43° C.; they also found that gas production was greater in lactose broth at 43 than at 37° C.

Harold (1937) tested modifications of two methods proposed by Wilson *et al.* (1935) and described in their work as 'Methods III and IV'.

Harold's 'method A' consisted in the direct inoculation of water samples into duplicate sets of MacConkey's broth, one set being incubated at 42° C., the other at 44° C. and all the positive 42° C. tubes were subcultured into citrate medium. (This method corresponds to Wilson's 'method III'.)

'Method B' (corresponding to Wilson's 'method IV') consisted in the inoculation of water samples into one set of MacConkey's broth with incubation at 42° C., each positive tube being subcultured into MacConkey's broth at 44° C. and into citrate medium at 37° C. Incubation at 44° C. was carried out in a water-bath for 48 hr.

It was noticed that with direct inoculation of water into MacConkey's broth at 44° C. the *Bact. coli* content was less in most cases than that found at 42° C.

In method 'B' the majority of the subcultures at 44° C. were positive and all proved to be of *Bact. coli* type I. In the course of a later and more extensive series of experiments with method 'B', this worker encountered twelve strains of '*Bact. coli* type II' which produced gas at 44° C. A question of terminology is here involved because '*Bact. coli* type II' which produces gas at 44° C. cannot exist in Wilson's classification; such a strain by definition becomes 'Irregular type II'.

A few I.A.C.[1] strains survived the high temperature, but as they were in every case associated with typical *Bact. coli*, it is not possible to say whether the former had, or had not, any hand in the production of the gas which was present.

Plating and differentiation from both the 42 and 44° C. positive tubes revealed an identical number of *Bact. coli* type I in each series. Harold therefore concluded that incubation at 44° C. after primary enrichment at 42° C. was specific for faecal *Bact. coli*.

Dodgson (1937) noticed that a large number of coliform strains in oysters were killed at 44° C. and that the test was very promising for the detection of typical *Bact. coli* in mussels.

Hajna (1937) described experiments on the fermentation by *Bact. coli* at 46° C. of a large number of carbohydrates and alcohols including all the

[1] This abbreviation is used throughout for the intermediate-*aerogenes-cloacae* group.

well-known 'sugars'. Most of them underwent fermentation. He stated that Eijkman's original medium was the poorest fluid culture medium for gas production at 46° C.

This investigator's other findings were that the presence of meat extract in the medium interfered with gas production at both 37 and 46° C.; that the presence of buffers increased gas production by permitting the decomposition of more carbohydrate before the toxic limit of acidity was reached and that the production of gas was greatly influenced by the type of protein in the medium.

Mackenzie & Hilton-Sergeant (1938) made comparative studies of faeces, using incubation temperatures of 37 and of 44° C. They formed a high opinion of the Eijkman test; to use their own words. 'There is no doubt that the isolation of *Bact. coli* type I subsequent to incubating at 44° C. is more certain than by selecting colonies from plates prepared after incubating in MacConkey broth at 37° C.' With regard to the use of the test for water analysis, they were of opinion that 48 hr. incubation should be allowed before reporting a negative result; that the percentage of false positives was negligible and that the test indicated the absence of dangerous pollution with greater certainty than did the 37° C. presumptive count.

They considered that there was a strong case for doing the Eijkman test in all laboratories concerned with water examination and suggested the laying down of new standards. They also stated, as touching the specificity of the test, that they had invariably recovered *Bact. coli* from their positive Eijkman tubes, no I.A.C. types having been isolated from them. These investigators thought that failure to isolate faecal *Bact. coli* from 37° C. presumptive tubes might occur because this organism had been overgrown by members of the I.A.C. group, whereas *Bact. coli* would readily have been detected by the Eijkman test.

Bardsley (1938), in an extensive research, compared the results of Wilson's 'Method IV' (subculture of all 37° C. positive presumptive tubes into MacConkey's broth at 44° C. and citrate medium at 37° C.) and of the usual method of plating and subsequent differentiation according to the *Ministry of Health Report*, no. 71 (1934).

From 550 water samples which contained coliform bacilli she isolated:

	Bact. coli type I	I.A.C. group
By M.O.H. method	417	213
By Wilson's 'IV'	446	449

and, in her opinion, these results showed that the plating method often failed to detect the presence of members of the I.A.C. group. The greater correspondence in the *coli* figures she attributed to the fact that *Bact. coli* I usually overgrew the I.A.C. organisms in MacConkey broth and had consequently the better chance of isolation.

In the 550 samples, *Bact. coli* type I only once remained undetected by Wilson's method, but was missed thirty times by the plating method.

The conclusions of Wilson *et al.* (1935) were confirmed, but Bardsley pointed out that the success of their method depended on the correctness of the assumption that a positive 44° C. test was indicative of the presence of *Bact. coli* type I; this question of specificity was therefore put to the test.

In all 1609 strains of *Bact. coli* type I were investigated and found to be 'Eijkman-positive', as were twenty-five strains of irregular type II; on the other hand, out of 1086 strains of I.A.C. organisms, only six were able to produce gas at 44° C.

The findings of Wilson *et al.* (1935) were again confirmed, and the truth of their assumption of the specificity of the test for faecal *Bact. coli* was clearly shown.

In the examination of faecal samples, the efficiency and specificity of method IV were again obvious.

Bardsley admitted that numerical estimations of *Bact. coli* type I would be upset if irregular type II—the only type which can be considered as a threat to the specificity of the test—were present, but considered that its occurrence was so rare as to be of little account in comparison with the degree of experimental error of the method. She concluded with the view that method IV was simpler, quicker and cheaper than the plating method.

Dodgson (1938) found that at a temperature of 44° C., all citrate-positive organisms in shellfish failed to produce gas, while the majority of typical *Bact. coli* were able to do so.

Further experiments at the Metropolitan Water Board (*Annual Report*, 1938) were carried out at 44° C. in a very carefully controlled water-bath with a maximum temperature variation of $\pm 0{\cdot}5°$ C. The tubes of medium were preheated before inoculation.

Three series of tubes were inoculated with water and incubated at 37, 42 and 44° C. respectively, with the result that the presumptive yield of faecal *Bact. coli* type I at 44° C. was only 63% of that obtained at 42° C. Although in comparison with the other two series the 37° C. set produced more positive presumptive tubes, there were fewer strains of *Bact. coli* I isolated from them. A trial of Wilson's 'method IV' revealed typicals in only a few more cases than the plating method, the results following fairly closely those of the 42° C. presumptive test. As far as specificity for *Bact. coli* was concerned, nearly all the 44° C. positive tubes were confirmed as containing *Bact. coli* in pure culture; on the other hand, out of 100 specimens, which, on subculture from presumptive positives, failed to give acid and gas at 44° C., no less than forty were shown to contain *Bact. coli* type I. This report concluded that direct inoculation of water samples into MacConkey broth followed by incubation at 44° C. gave results inferior as regards recovery of *Bact. coli* type I, to the method of incubation at 37 or 42° C. followed by plating. The plan of subculture into MacConkey's broth at 44° C. showed, however, slightly better results than the plating method. On the principle that there is no need to look for I.A.C. organisms when *Bact. coli* has been shown to be present, this

report recommended that all positive presumptive tubes should be subcultured into MacConkey's broth at 44° C., followed by plating and identification of only those 37° C. tubes which proved to be 'Eijkman-negative'.

Hajna & Perry (1938) carried out an investigation in which they compared the results obtained with their own modified Eijkman medium (1933) (containing glucose) with those obtained after substituting lactose for glucose. They also tried the effect of the addition of 0·1% of sodium formate.

Incubation was at 46° C., and parallel tests were done at the same time with 'Standard Methods' lactose broth at 37° C. The authors' medium, using lactose, resulted in the recovery of more *Esch. coli* than any of the other media, 'Standard Methods' lactose broth at 37° C. proving only slightly more than half as efficient. The addition of sodium formate to the authors' medium decreased the yield of *Esch. coli* when the medium contained lactose, but improved the yield when the medium contained glucose.

Clegg & Sherwood (1939), working on shellfish, used a water-bath with a special mercury-toluene thermo-regulator permitting a maximum variation of temperature of only ±0·1° C.

They performed comparative tests of colonies from plates made from polluted mussels, using MacConkey's broth at incubation temperatures of 37, 41, 42, 43, 44, 45 and 46° C. The tubes were not preheated before inoculation and experiment showed that the medium reached 44° C. after the tubes had been in the bath for 9 min. 59·2% of citrate-positive strains produced acid and gas at 37° C., this figure gradually dropping to 0·5% at 44° C., so that it was clear that the high temperature practically eliminated these organisms. Among sixty-nine citrate-negative strains which fermented lactose at 44° C., every one was methyl-red-positive and Voges-Proskauer-negative, while all but three produced indole. Four citrate-positive cultures were encountered which gave positive Eijkman tests. These workers concluded that, owing to its high degree of specificity for faecal *Bact. coli*, the 44° C. test was of great value in the examination of shellfish. They pointed out that the problems in this field are very different from those met with in water analysis because the I.A.C. group may be present in abundance in shellfish when they are quite free from faecal pollution. They reported an almost perfect negative correlation between the Eijkman and citrate tests and observed that temperatures above 44° C. were detrimental to the growth of *Bact. coli*.

Raghavachari & Iyer (1939) undertook an investigation of water samples using the modified dilution method described by Wilson *et al.* (1935). They incubated the tubes at 37° C. and subjected one positive tube from each water sample to the usual plating and differential tests—including the 44° C. MacConkey test.

In addition, every positive 37° C. tube was subcultured directly into MacConkey's broth at 44° C. and into citrate medium at 37° C. (Wilson's method IV); all the 44° C. positives obtained in this way were plated out and subjected to differential tests.

The results obtained were most discouraging. In Wilson's method IV, as well as in the differential tests from the 37° C. positives, about 75% of the *aerogenes*-like cultures were found to give positive results at 44° C. This finding was a bomb-shell after the work of Wilson and of Bardsley, but it should be noted that the positive 44° C. tubes were plated out on MacConkey's agar and incubated at 37° C. This means that if organisms of the I.A.C. group were viable—though not necessarily growing—in the broth at 44° C., they would be revealed on the plate at 37° C. This factor, the authors say, might account for the above findings. Owing to the violent clash between their results and those of Wilson, Raghavachari & Iyer checked their technique very carefully and found it to be in every way correct. Besides this, they sent some of their 'Eijkman-positive' *aerogenes* strains to Prof. Wilson who reported that their behaviour was the same in his hands.

The main contention of the authors—'that some strains of *Bact. aerogenes* isolated from Indian waters produce acid and gas in MacConkey at 44° C.'—was therefore proved correct. It will be remembered that Brewster (1929) and Burke-Gaffney (1932) had anticipated this conclusion.

At about this time there appeared a new edition (*Report*, 1939) of the *Ministry of Health Report*, no. 71, and in this the Eijkman test received a partial official sanction.

The specificity of the test for faecal *Bact. coli* and its rapidity were praised by this *Report*, and the importance of constancy of temperature was stressed. It was pointed out that, should the temperature rise to 45 or 46° C., a certain proportion of strains of true faecal *coli* would fail to produce gas, while, should the temperature drop to 42° C. some of the I.A.C. group would produce gas.

The report then described Wilson's 'Method No. IV' but suggested the method proposed by Mackenzie (1938) which has already been quoted in this survey, namely, subculture of all positive 37° C. tubes into MacConkey broth at 44° C.; then, in the case of negative 44° C. tubes only, examination of the corresponding 37° C. tubes by the ordinary plating and differential methods.

Duplicate direct inoculation of water with incubation at 37 and at 44° C. was also suggested, or even a single set at 44° C. when a rapid result was desired. The report stated that the information was conflicting as to whether a higher count was given by direct inoculation at 44° C. or by subculture into 44° C. broth from 37° C. positives.

As far as the actual performance of the test was concerned, preheating of the tube to 44° C. before inoculation was recommended.

The report concluded that the available data justified the use of the test in routine work.

Ferramola, Gelstein & Dolcetti (1939), encouraged by the results obtained by Bardsley (1938) with Wilson's method IV, tried the method on waters in the Argentine. They tested by plating and the usual differential methods, many of the positive Eijkman subcultures which had been made from the

37° C. presumptive tubes. Over 93% showed the presence of *Esch. coli* faecal type I or II. It would be interesting to consult their original paper, which unfortunately is inaccessible, to see if they found 'faecal type II'[1] present *alone*, as according to most recent observers, this type does not give gas at 44° C. Further verification of the efficacy of the method was undertaken upon 1000 coliform strains derived from sewage and faeces, and a very satisfactory correlation was obtained between the results of the Eijkman test and those of the usual typing methods.

Perry (1939), in direct contradiction of Bardsley (1938), held that the greatest value of the Eijkman test appeared when working on samples with a high content of *aerobacter* strains because these were apt to overgrow *Bact. coli* at 37° C.

Hajna & Perry (1939) set out to discover whether temperatures of less than 46° C. could be satisfactorily employed as a selective test for *Bact. coli*; also, to determine the relative values of MacConkey's broth and of their own modified Eijkman medium (Perry & Hajna, 1933) at 44° C. This modified Eijkman medium was varied experimentally by substituting lactose or mannitol for the usual glucose. Using known strains, these workers found that the I.A.C. group of organisms all produced gas from glucose, lactose and mannitol at 42° C., that many did so at 44° C. and a very few at 46° C. All the *Escherichia* strains fermented mannitol at 46° C.

In the comparative tests upon MacConkey's broth and the authors' medium, two sets of each medium were used, one of which sets was incubated in an incubator, the other in a water-bath.

It is noteworthy that these authors still favour the use of an incubator in preference to a water-bath. They believe that the time lag (which is much more pronounced in an incubator) before the tubes reach full temperature is an advantage, as it allows the organisms to acclimatize themselves.

Judged upon the number of positive results given at 44° C. by *Escherichia* strains, MacConkey's broth was slightly inferior to the authors' medium, and the set of tubes which had been incubated in a water-bath showed still more failures. Many *aerobacter-aerogenes* strains were found to grow at 44° C., but MacConkey's medium inhibited them more strongly than did the other medium. It was noticed that the positive MacConkey tubes showed much less gas than those of the 'modified Eijkman' and that very few of the cultures in MacConkey's broth were viable after 48 hr., but that the organisms could be isolated even after 96 hr. from the authors' medium.

Perry & Hajna considered that their medium was better than MacConkey's and believed that this superiority was due to the lower concentration of carbohydrate and to the presence of buffers. They recommended the use of a temperature of 45·5–46° C. for the Eijkman test.

Ferramola & Monteverde (1939), working upon organisms obtained from Buenos Aires sewage, demonstrated that the 44° C. MacConkey test was highly specific for typical *Bact. coli*.

[1] "Irregular II" is probably meant here.

Raven, Peden & Wright (1940) have done fairly extensive work with Wilson's method IV, and their remarks will be quoted in some detail.

They found a large number of instances in which citrate utilizers grew at 44° C. This is a disquieting contradiction of recent work by others, as will be apparent from what has gone before.

For the Eijkman test, MacConkey's broth was used, the tubes being warmed before inoculation, and incubated in a water-bath at 44° C. In most cases the test was complete in 24 hr., and out of 134 positive tubes examined, 130 yielded *Bact. coli* in pure culture; two strains of *Bact. aerogenes* which were Eijkman-positive occurred in this series.

These workers consider this method very satisfactory for water analysis.

Their next investigation consisted in performing the presumptive test at 44° C. (by Wilson's method IV) in parallel with the usual 37° C. presumptive test. The results were as follows:

Bact. coli absent both tests	194
Bact. coli count the same in both	55
Discrepancies	80
Samples tested	329

The eighty discrepancies were made up as under:

Bact. coli present at 37° C. absent at 44° C.	31
Bact. coli absent at 37° C. present at 44° C.	22
Count at 37° higher than at 44° C. ...	15
Count at 44° higher than at 37° C. ...	12
	80

Most of the discrepancies are explicable as due to the experimental error of the method, but some are greater than this explanation warrants.

A total of 183 tubes of citrate which showed visible growth were examined and found to contain *Bact. coli*, all of which had been previously revealed by the 44° C. test, but none of these strains grew in citrate when they were retested in pure culture. The authors thought that the turbidity might have been due to organisms not of the *coli* group, or that possibly it occurred because they had inoculated their citrate tubes by loop instead of by straight wire; however, parallel inoculations of citrate by these two ways were found to produce very little difference in results.

Harding (1940) states that the test has been found of little value in Derbyshire, since in this region many I.A.C. organisms also give positive results. He has performed parallel inoculations at 44 and 37° C. and found that two waters in particular always give positive results at 44° C. On plating, faecal *coli* are never found, but intermediate I and *aerogenes* I are present and both confirm as Eijkman-positive.

This observation is important as it is the first seriously adverse report in this country upon the specificity of the test.

Ferramola (1940) has examined a large series of samples by Wilson's method IV.

He obtained from his 37° C. series of positives, five coliforms which failed to ferment lactose at 37° C., but which produced gas in MacConkey at 44° C. Ferramola's 44° C. series was inoculated directly from the positive 37° C. tubes and was checked by plating and the usual differential tests including a 44° C. MacConkey tube. Of 309 positive tubes examined, 294 yielded *Bact. coli* type I, three *Bact. coli* type II, six gave I.A.C. organisms, and from the remaining six no growth was obtained. That is to say, 95% of the positives contained faecal coli.

A further series of cultures obtained from sewage and faeces was differentiated, and of 963 strains classified as *Bact. coli* I, six only failed to produce gas at 44° C. and should presumably be classed as irregular I. Of 105 other organisms, not a single one was positive at 44° C.

This work gives the impression of being very carefully performed, and the author justly forms the opinion that the test is rapid and accurate. He points out that the Wilson method enables a confirmed and differential coliform count to be completed in the same time as that needed by American standard methods for the coliform count alone, and that it has great advantages in that it dispenses with plating and microscopical examination.

Raghavachari & Iyer (1940) refer to their previous paper (1939) in which they concluded that the 44° C. test could be considered specific for distinguishing between faecal and non-faecal types only if it could be proved that 44° C. positive *aerogenes*-like organisms found in water were normal intestinal organisms.

It should be noted, however, that this statement applies to Indian waters in which so many of these recalcitrant *aerogenes* strains have been found.

In support of their view that *aerogenes* organisms may be true faecal types they quote Minkevich & Rabinovich (1936) for the opinion that all soil strains of coliforms are derived by adaptation from faecal *Bact. coli*, and Horwood & Webster (1937) for the suggestion that *aerogenes* types form the normal flora of the small intestine but are transformed into *Bact. coli* upon arrival in the large intestine.

The workers further quote Sen (1937) as reporting that many strains of *aerogenes* isolated from human faeces give a positive Eijkman reaction and have set out to reinvestigate this problem for themselves.

Fresh specimens of faeces from fifteen healthy individuals were tested in two ways. The first method consisted in direct plating on to two sets of MacConkey plates, one set of which was incubated at 37° C. and the other at 44° C. Colonies were picked and underwent the usual differential tests including the 44° C. MacConkey test for which water-bath incubation was used.

The second method was an enrichment method and consisted in emulsifying each specimen in peptone water. 1 c.c. of the emulsion was added to each of two MacConkey broth tubes, one of which was incubated at 37° C. and the other at 44° C.

Two MacConkey plates were then made from each of these tubes, one being incubated at 37° C., the other at 44° C., and the usual examination of colonies followed.

The results were highly significant. Out of 478 organisms studied, thirty-one were of *aerogenes* I type, and no less than twenty-four of these were Eijkman-positive. These strains were frequently retested at 44° C. and all were consistently positive; similarly, the seven Eijkman-negative strains remained negative.

The authors conclude that present ideas as to the significance of *Bact. aerogenes* in water analysis must be revised, and they advance the hypothesis that all coliforms are faecal in origin, having changed their properties when removed from their normal habitat.

Banerjea & Sen (1940) investigated faeces from Indian patients. They compared the results using MacConkey broth at 44° C. with those using Perry & Hajna's (1935) buffered dextrose medium incubated at 46° C. This seems a curious basis for comparison, especially in view of the general acceptance of the lower temperature for some years past. They found that four out of nine *aerogenes* strains were Eijkman-positive, but it is difficult to evaluate their results for the reader is not left absolutely clear as to their procedure.

Taylor (1941), in the course of his work upon waters from lakes and streams in the Lake District, has tested all cultures for growth at 44° C. in MacConkey. It is worthy of note that the medium was placed in the water-bath for some hours to bring it up to temperature before inoculation which was performed with the tubes in situ. The water-bath permitted a temperature variation of $\pm 0{\cdot}4°$ C.

Taylor's results are not so specific as those of Bardsley (1938), for of thirty cultures of *Bact. aerogenes* I, six were 44° C.-positive and one strain of intermediate I also gave positive results. This worker states that these cultures were retested and that there is no doubt that strains of *Bact. aerogenes* do exist which are positive at 44° C.

Clegg (1941), examining water samples, compared faecal *coli* counts obtained by direct and secondary incubation at 44° C. (i.e. Wilson's method III versus method IV). The 44° C. tubes were not heated prior to inoculation.

It is unnecessary to describe the technique used, since these methods have already been fully dealt with under Wilson's work, but an unusual detail should be mentioned. Since doubt has been expressed by some workers as to whether minimal inocula will suffice for tubes to be incubated at 44° C. (Harold, 1935, Raven *et al.* 1940), subculture into MacConkey tubes was performed with a triple loop (three on one wire).

From North Wales 837 samples of different types of water were examined, and 79·5% of those containing coliforms were found to contain faecal *Bact. coli*. 63·3% of the 37° C.-positive tubes were positive after 24 hr., and confirmatory tests showed that 76·4% of these contained either *Bact. coli* I or Irregular II. Of the 36·3% of the tubes which were positive only after 48 hr., but 17·1% contained these organisms.

Forty-six cultures of the Irregular I type (M.R.+, V.-P.−, citrate −, indole +) were isolated, that is, strains which failed to produce gas at 44° C. in the ordinary confirmatory test; however, when they were isolated in pure culture they did so and continued to do so on repetition. This worker claims that these results were not due to any deficiency of technique, but suggests that the enzymes may need time to become adapted to the high temperature, or alternatively, that the presence of an inhibitory organism in the subculture from the primary growth may exert an effect.

A table is given showing much higher viable counts for *Bact. coli* incubated in broth at 37 than at 44° C., and from this Clegg concludes that 44° C. is not as suitable a temperature for this organism as is 37° C.

This observation contradicts that of Wilson *et al.* (1935) who, in his experiments with milk, found that the higher temperature was not at all inhibitory to *Bact. coli*; the opposite was in fact the case.

Other cultures occurred which gave positive reactions both at 44° C. and in citrate, but when subsequent subcultures were made, all (except one strain of *Bact. aerogenes* I) proved to be citrate-negative.

The results of confirmatory tests on the 44° C. primary positive tubes were as follows. Of these, 2·4% failed to give typical *Bact. coli* colonies on the E.M.B. plate; 1·7% gave no growth and 0·7% showed atypical colonies. Of these, three were small dark colonies of a coccus, three were of Irregular I type and one confirmed as *Bact. coli* I. All the remainder failed to give a positive 44° C. test on repetition.

Speaking of those tubes which gave no growth on the plates this worker suggests that either certain cultures of *Bact. coli* will not remain alive in MacConkey broth at 44° C. for 48 hr. or else that *anaerobes* were responsible for the production of gas in the primary tubes.

The comparison of the relative efficiency of methods III and IV showed that in no group of water samples did the figures obtained by method III exceed those of method IV. Taking the whole of the results the difference between the average probable number of *Bact. coli* yielded by the two methods was not great (III : IV = 72·6 : 100).

Clegg does not think that method III should be discarded, even though it is obviously less efficient, because first, in 25% of samples it gave a higher count than method IV, and secondly it is much quicker. Since speed is of importance in reporting he thinks the two methods should be run in parallel in order to secure the highest efficiency.

The precise comparative results of the two methods were: number of samples showing *Bact. coli* by one or both methods, 413. Number of samples in which the probable number of *Bact. coli* differed by the two methods, 353. The proportion of samples in which the results differed significantly was small.

A review of the literature seems to indicate that the Eijkman test is an extremely valuable addition to the procedures used in water examination because of the high degree of specificity for faecal *Bact. coli* which it displays.

Although papers may continue to appear which question this specificity, it is evident that, as the years have passed, the voices raised in adverse criticism have gradually become fewer, though it must be admitted that of late years in India and more recently at home, considerable numbers of Eijkman-positive strains of *aerogenes* are being isolated.

In this connexion it is well to mention that, particularly in some of the earlier work, the specificity of the test may have been impugned on insufficient grounds. It is absolutely necessary when examining the contents of a primary positive 44° C. tube to subject the strains isolated to a further 44° C. test. If this is not done, organisms which have been merely living (without producing any gas) in the primary tube may appear to be 44° C.-positive strains. In many of the papers there is absolutely no indication as to whether this was done or not, and the writer is convinced that some of the adverse comments of the earlier workers were due to this omission.

Certain factors clearly emerge from a study of the literature as being of vital importance—temperature and composition of medium.

Of the first, it is strange that it took so long before the suitability of the 46° C. temperature came to be questioned, since as early as 1908, Barber had written: 'It is certain that in this species (*B. coli*) there is only a narrow margin between the point at which the maximum rate of reproduction ceases and the thermal death point of a large proportion of the individuals. Even at 45° growth was less regular, and there is an increase in the proportion of bacteria which fail to grow.

It is interesting that 44° C. has now become established as the optimum temperature.

In the matter of the composition of the medium, the observation that a toxic level of acidity was reached therein and that evil effects resulted, pointed the way for two improvements—the reduction of the percentage of carbohydrate and the introduction of buffers. Although no buffers, other than those present in peptone, are employed in MacConkey's broth which is now successfully used for the test, the work on this factor was instructive.

Two other stones in the foundations of the present reliability of the test have been the substitution of lactose for glucose originally used by Eijkman and the recognition of the necessity for a perfectly controlled temperature which has found expression in the almost universal employment of water-bath incubation.

Part II. Experiments

A. *General plan and scope of the work*

This paper was designed as a survey of the work of others, yet it is perhaps worth while to record the results of the examination by the writer of a series of 104 water samples, wartime shortage of staff and materials having cut short further examinations.

In May 1940 this laboratory began to use the 44° C. MacConkey test by

https://doi.org/10.1017/S0022172400012638 Published online by Cambridge University Press

direct inoculation (i.e. Wilson's method III), but, owing to the unreliability of citrate for indicating the I.A.C. group, this part of Wilson's method was omitted. It was therefore decided: (1) to investigate the specificity of 44° C. incubation for *Bact. coli* type I, and (2) to inquire whether the more accurate information as to the presence or absence of this organism in water samples is given by the use of Wilson's method III or by plating and differential tests from the 37° C. presumptive positive tubes. Most workers agree that citrate is unsatisfactory as a differential medium, and it therefore seemed a good opportunity to try cellobiose fermentation which some have claimed to be an efficient substitute.

The large number of cultures to be tested also offered an opportunity to compare the methods of O'Meara and of Barritt for the Voges-Proskauer test, and it is hoped to describe the results of this and of the cellobiose inquiry in separate communications.

Great care was taken to make all media for the differential tests of the most suitable materials and to perform those tests by up-to-date methods.

B. *Methods and media*

All media were made according to the specifications given in the *Report* (1939), and any variations in materials or preparation are noted in the ensuing pages.

MacConkey agar

During the first 393 cultures the MacConkey agar used was Grunbaum and Hume's modification which differs from the usual formula only in the addition of crystal violet in 1 in 100,000 concentration and in having half the usual quantity of neutral red. This medium was at the time in routine use in this laboratory, but for the remainder of the work standard MacConkey agar plates were used.

Methyl-red test

This was performed upon 3-day cultures in glucose-phosphate medium by adding to each 0·25 c.c. of 0·04% methyl-red solution. From a few comparative tests it seemed that if the medium were made with 'Bacto' peptone, the results were more clear-cut than if 'Evans's' peptone was used. Accordingly, the medium for this test was made with 'Bacto' peptone for the first half of the work.

While there is no doubt that this peptone is quite suitable for the test, it was discovered later that the manufacturers (Difco Laboratories) recommended their 'Proteose' peptone as being the more suitable for this and for the Voges-Proskauer test.

This claim was supported by Skinner & Brudnoy (1932) who stated that proteose peptone was the only one which gave results as satisfactory as those given by Witte's peptone which was that originally specified by Clark & Lubs (1915). 'Difco Proteose peptone' was therefore used during the remainder of

this investigation, and all the methyl-red results which were 'doubtful' or which were for any reason suspicious, were confirmed by its use.

Voges-Proskauer reaction

The test was performed upon both 2- and 3-day cultures by O'Meara's (1931) method; also upon 2-day cultures by Barritt's (1936) method modified to give greater sensitivity by addition of a knife-point of creatine and 2 drops of 2% ferric chloride solution. To achieve the maximum number of positive results, the O'Meara test was read after 4 hr. standing and the Barritt test after 1 hr. (Batty-Smith 1941).

Citrate utilization test

Inoculation was carried out with a straight wire as recommended by the *Report* (1939) and brom-thymol blue in 0·008% concentration was added as an indicator. Following the practice of Wilson *et al.* (1935) the cultures were examined for growth after 5 days.

The reaction of the medium was adjusted, as recommended in the *Report* (1939), to *p*H 6·8; but after the first 250 cultures this was altered to *p*H 6·4–6·6, as recommended by Harold (1937). As this alteration resulted in an initial pale apple-green tint, the change to blue-green was more easily detectable. Harold states that the growth-promoting qualities of the medium are unaffected by this change in reaction.

Ruchhoft *et al.* (1931) advised that all citrate tubes should be preserved at room temperature after being read, since they found that strains could be recovered from them after long periods and that this could be done even in the case of *Bact. coli* when there was no apparent growth at all.

All citrate tubes in the present investigation were stored in this way, and proved very useful for confirmatory re-examinations.

Indole test

This was performed upon 3-day cultures by the xylene extraction method of Happold & Hoyle (1934), and it was decided to experiment with shaking more vigorous and prolonged than the usual 'good shake' given by hand.

Accordingly, 2 c.c. of xylene, as advised by these workers, was added and the cultures shaken for 5 min. in a mechanical shaker. At the end of this period, 1 c.c. of Ehrlich's reagent was run on to the surface of the mixture immediately (without waiting for the liquids to separate), with the result that the pink colour obtained in positive cultures by the usual hand shaking gave place to a deep red.

From these experiments it was concluded that vigorous and prolonged shaking is vital to the satisfactory performance of the indole test, and it seems curious that its importance is not stressed in any of the textbooks.

Comparative experiments with 10 min. as against 5 min. shaking were carried out, but the longer period apparently conferred no benefit.

The often recommended addition of 1 c.c. of a saturated solution of potassium persulphate was also tried, but had not the slightest good effect on the test when performed in this way.

In the hope of obtaining still stronger positives, 'Bacto Tryptone' (a special peptone with added tryptophane) was used to make the peptone water for this test. It appeared to give slightly stronger positives than did 'Bacto' peptone water.

The 44° C. MacConkey test

MacConkey broth was inoculated and immediately placed in a water-bath at 44° C., the test being read after the elapse of 48 hr. The medium was not heated up before inoculation.

A result was not held to be positive unless the curved end of the Durham's tube was filled with gas.

The water-baths used were of Messrs Hearsons' usual type (gas-heated with temperature regulation by capsules) and maintained a temperature which varied between 43·5 and 44·5°. The thermometers in use were compared with a certificated instrument and found to be accurate.

Fermentation of glucose and of lactose

The results were read after 2 days and after 5 days, a positive result being recorded when there was sufficient gas to fill the curved end of the Durham's tube.

Fermentation of cellobiose

The medium was made up in peptone water in exactly the same way as the other 'sugars' except that cellobiose in a concentration of only 0·25% was used. Incubation was carried out at 37° C. and the test read after 2 and after 5 days. When gas production took over 2 days to occur, 'late' fermentation was recorded (Batty-Smith, 1942).

Gelatin liquefaction

This test serves only to distinguish between *Bact. aerogenes* and *Bact. cloacae*, a distinction which is without object in routine water bacteriology, since the sanitary significance of these two organisms is practically the same. For this reason the test was not applied in the present work.

The principles governing the selection of colonies from plates

If all the colonies on the plates were of typical coliform type, two or three, if possible of different appearance, were picked.

As regards colonies of atypical appearance, these are of three kinds:

(1) Those produced by organisms not belonging to the coliform group non-lactose-fermenters and alkali-formers.

(2) Typical coliform colonies which have been deprived of their power to produce a red colour through the presence of contaminating alkali-forming organisms. These often produce sufficient ammonia to neutralize the acid

formed by colon bacilli. *Bact. alkaligenes* and *Bact. fluorescens* are met with in waters and are strong alkali-formers.

(3) Colonies which owe their atypical appearance to a temporary slowing of their action on lactose.

In view of the possible masking of typical coliform colonies in the two ways just described, it was decided, to avoid missing any genuine lactose-fermenters, to pick atypical colonies so long as they proved, when stained by Gram's method to consist of Gram-negative bacilli of coliform-like appearance.

C. *Technique of examination of each water sample*

The routine examination

The routine examination of water samples is carried out in this laboratory according to the technique of the *Report* (1939).

For each sample, one tube containing 50 c.c. of double strength MacConkey broth is inoculated with 50 c.c. of water; five tubes, each containing 10 c.c. of the same broth, each receive 10 c.c. of the sample and finally, five tubes each containing 5 c.c. of single strength broth are each inoculated with 1 c.c. of the sample. Smaller quantities of water are never used here, since the total of eleven tubes allows of a presumptive coliform count up to 180 per 100 c.c. of the sample.

The tubes are incubated at 37° C. for 48 hr., and the results read from 'probability tables'. The presence of sufficient gas to fill the curved end of the gas tube is considered to indicate a positive result.

A duplicate series of tubes is inoculated and placed immediately in a water-bath at 44° C., the results being read after 48 hr. These tubes are not preheated before inoculation.

Where a sample is known or expected to be of fairly good sanitary quality, the 1 c.c. quantities of water are omitted.

Plating and differential tests

From the 37° C. *series.* The two tubes which showed the presence of gas and contained the smallest quantity of the original sample were chosen for plating.

It was soon noticed on many occasions that, when plates were laid aside at room temperature after 24 hr. incubation at 37° C., additional varieties of colonies (often lactose-fermenters) made their appearance. Therefore it was thought advisable to incubate the plates for 48 hr. at 37° C. to ensure that all lactose-fermenters had 'come up' at the time of subculturing. Usually three colonies, including colonies of atypical appearance, were picked from each plate and put through the usual differential tests including glucose, cellobiose and a 44° C. MacConkey broth tube.

From the 44° C. *series.* The same procedure was adopted except that only one primary positive tube was plated out. The colonies from this series were nearly always typical, *Bact. coli* I being usually present in pure culture, so that two colonies only were picked from each plate.

D. *Analysis and discussion of experimental findings*

General comment

As far as possible each organism was named according to Wilson's classification. There were many that could obviously not be included as members of the coliform group, but in other cases it was doubtful whether the organism should be so included. In yet other cases, the original results of some of the differential tests were equivocal, and though many of these doubtful results upon repetition of the tests became definitely positive or negative, there were some which remained inconclusive.

How to classify these strains was a continual source of worry; and as a general rule, any strain which showed a characteristic grouping in the methyl-red, Voges-Proskauer, citrate and indole tests was set down as a member of the coliform group even if it was a late or non-lactose fermenter. Well over half the number of intermediate and *aerogenes* strains failed to ferment lactose promptly and there were also a few strains which, though they gave the general reactions of faecal *Bact. coli*, produced only a small bubble of gas in MacConkey broth at 44° C., and either failed altogether to ferment lactose or else fermented it late or without gas production.

In these borderline cases, the strain was given a name after consideration of all the circumstances and of the results of repetition, but fortunately the number was not great enough to have any appreciable influence on the conclusions.

Since writing these words a paper by Stuart, Mickle & Borman (1940) on the grouping of slow lactose-fermenting coliforms has come under notice. These workers define a typical coliform as one which produces 20% or more gas in 48 hr. at 37° C. They describe as 'aberrant coliforms' those which do not comply with this definition and suggest a classification of them into four groups:

(1) 'Micro-aerogenic' or genuine slow lactose fermenters.

(2) 'Pseudomicro-aerogenic' coliforms which are slow lactose fermenters at 37° C., but which form abundant gas in lactose at 20° C.

(3) 'Papillae-forming' coliforms which show the characteristics of *Bact. coli mutabile*.

(4) 'Anaerogenic' coliforms which produce acid only in lactose in 1–7 days.

They also believe that genuine non-lactose-fermenting coliforms exist. This work promises to have far-reaching results and seems to indicate that the fermentation of lactose has hitherto been given too much importance as a qualification for membership of the coliform group.

Examination of 44° C. *series*

It was rarely that more than one kind of colony grew on the plates from this series, and the great majority of tubes apparently contained *Bact. coli* I in pure culture. One strain produced large mucoid colonies which by the following day had grown into heaped up, creamy masses the size of a sixpence.

It is difficult to explain why three tubes gave no growth on the plates, but it is noticeable that the count was very low in each case, that is, of the order of one to three organisms per 100 ml. The absence of growth in the first of the cases is the more curious as the 37° C. count was 50 and *Bact. coli* I was recovered from the 37° C. positive tube, also the count by subculture into 44° C. MacConkey (Wilson's method IV) was 11. It is difficult to believe that *Bact. coli* I was not present in the direct 44° C. series here.

In the second case, the 37° C. count was 50, the count by method IV was 0, and *aerogenes* I and Intermediate I were alone recovered from the 37° C. series.

In the third sample, the counts at 37° C. and by method IV were both 0, so that in this case and the preceding one *Cl. Welchii* may have been the cause of the original presumptive reactions at 44° C.

The sample which gave only a growth of Gram-positive cocci from the 44° C. series should be considered here. The direct 44° C. count was 8; by method IV, 5; and at 37° C., 35, *Bact. coli* I being recovered from the 37° C. tubes. Again, it is hard to believe that *Bact. coli* I was not responsible for the positive reaction at 44° C.

Table 2. *Analysis of sixty-four positive* 44° C. *tubes*

No. of tubes:	
Confirmed for *Bact. coli* I	56
„ „ *Bact. aerogenes* I (alone)	1
„ „ Irregular I (alone)	2
„ „ Irregular II (alone)	1
„ „ *Gram-positive cocci* (alone) ...	1
Giving no growth on plates	3
Total positive tubes examined	64

It is possible that in this sample and the first one, *Bact. coli* would have been recovered had the plates been made after 24 hr. incubation, for some strains may be unable to live for 48 hr. at 44° C. in the presence of the greater rise of acidity which takes place during the second 24 hr.

The two cultures of *aerogenes* type I failed to confirm as Eijkman-positive. They must have remained viable at 44° C. while the *Bact. coli* died out and have grown on the plates at 37° C. Alternatively, the original reaction was caused by *Cl. Welchii*. This seems a probable explanation because the 44° C. direct count was only 2, the 44° C. indirect count was 0, while at 37° C. the coliform count was over 18 and *aerogenes* strains only were recovered.

Of the twelve cultures of Irregular type I, five proved on retest to be Eijkman-positive; they were accordingly transferred to the total of *Bact. coli* type I. One of these strains was a non-lactose-fermenter at 37° C. and was confirmed as such, but on reinoculation into MacConkey broth at 44° C. produced a small bubble of gas.

The presence of the Irregular type I strains is easily accounted for. In every case except two, *Bact. coli* type I was present as well and could cause gas production while the Irregular type I organisms merely remained alive.

This close association with true faecal coli was noticed by Bardsley (1938) from which she concluded that this type was of faecal origin.

In one of the two samples in which Irregular type I was apparently present alone, it may be that mere chance resulted in the picking of colonies of this organism rather than of *Bact. coli*, for it is noticeable that this sample had a 37° C. count of 90 (which proved to consist chiefly of *Bact. coli* I) a direct 44° C. count of 8 and an indirect 44° C. count of 7. It is difficult to resist the conclusion that *Bact. coli* I was present in the original presumptive tube and would have been recovered had more colonies been picked.

A possible explanation of this result is that the sample originally contained large numbers of organisms of Irregular type I accompanied by relatively few *Bact. coli* which then overgrew the irregulars in MacConkey broth at 37° C. so that these were not revealed by differentiation. On the contrary, in the 44° C. series, large numbers of the irregulars must have remained viable and have outnumbered the *Bact. coli* on the plate, thus giving the irregulars a better chance of isolation.

In the other sample which was confirmed for Irregular type I alone, the 44° C. count was 1 and the 37° C. was 0, so that here there is no reason for presuming the presence of *Bact. coli*.

Table 3. *Analysis of* 123 *cultures from* 44° C. *tubes*

Cultures	Original results	Eijkman + after repetition of tests
Bact. coli type I	107	112
Bact. aerogenes type I	2	—
Irregular type I	12	—
Irregular type II	2	2
Total	123	114

A further possibility is that these irregulars were true faecal coli which had lost their power to ferment lactose at 44° C. on subculture as reported by Williams *et al.* (1933).

The two cultures of Irregular type II were retested for indole production and confirmed as negative.

The results after repetition shown in Table 3 indicate that the only two types which produced gas at 44° C. were *Bact. coli* type I and Irregular type II. As shown in Table 2, fifty-seven tubes confirmed for these two types out of sixty-four examined, so that the method shows an efficiency of 89% for the detection of *Bact. coli* I and Irregular II. If, in the light of the above discussion, we presume the presence of *Bact. coli* in the first of the samples showing no growth, in that confirmed for Gram-positive cocci and in the first of those confirmed for Irregular I alone, then sixty tubes would be considered as true positives and the efficiency shown would be 93·75%.

Examination of the 37° C. *series*

On repetition of certain of the tests:

(1) The three late lactose-fermenting strains of *Bact. coli* were confirmed as Eijkman-positive.

(2) A citrate-positive strain of *Bact. coli* I was confirmed as positive with heavy growth and alkaline reaction.

(3) Of the two doubtfully Eijkman-positive, partial lactose-fermenting *Bact. coli*, one again produced only a small bubble of gas at 44° C. and the other proved Eijkman-negative.

(4) All three cultures of Intermediate type I continued to ferment lactose at 44° C. when retested.

(5) Of three cultures of *Bact. aerogenes* type I, one failed on repetition to produce gas in MacConkey broth at 44° C., but the other two confirmed as 44° C.-positive on retest. Further, plates were made from the citrate tubes, colonies were picked and put through the differential tests when gas production at 44° C. was again present, though it was noticed that the amount of gas produced was consistently small and filled only the curved ends of the gas tubes.

(6) The single atypical organism which appeared on first isolation to be 44° C.-positive, failed on repetition of the test.

(7) A single strain only of Irregular type II was isolated and proved, when re-examined, to be definitely indole-negative.

Table 4. *Analysis of* 243 '*Eijkman-positive' cultures isolated from the* 37° C. *series*

	Original results	Eijkman + after confirmations
Bact. coli type I	220	229
Bact. coli type I late-lactose-fermenters	3	4
Bact. coli I Eijkman ±; acid only in lactose	2	1
Bact. coli I Eijkman +, lactose −	5	3
Intermediate type I	3	3
Bact. aerogenes type I	3	2
Irregular type II	1	1
Atypical non-lactose-fermenters	1	0
Total	238	243

In addition to all these cultures which were Eijkman-positive on first isolation, all strains of Irregular type I were retested in MacConkey broth at 44° C., and nine of them which were proved to be positive were added to the revised total of *Bact. coli* type I. One was a late lactose-fermenter and so was added to the total of 'late-lactose-fermenting *Bact. coli* I'.

Taking the figures after confirmation (Table 4) the specificity of the test is again strikingly evident since 98% of the 44° C.-positive cultures were either *Bact. coli* I or Irregular type II, the remaining 2% being composed of the intermediate and *aerogenes* strains.

Comparison of the relative efficiency for the detection of Bact. coli *I of the direct* 44° C. *method and the* 37° C. *presumptive coliform test with plating and differentiation*

Taking the results shown in Table 6, it will be seen that out of sixty-eight samples in which *Bact. coli* I was shown to be present it was detected by the 44° C. method in thirteen samples in which it was not revealed by isolation

from the 37° C. series; on the other hand, in nine samples the 44° C. method failed to detect *Bact. coli* when it was demonstrated in the 37° C. series.

Since in eight of these nine samples the counts at 37° C. were not higher than 3 per 100 ml. the sampling error of the method was very likely responsible, but in the ninth sample which gave a 37° C. count of 20 another explanation is demanded. Of the two 37° C. primary positive tubes plated, one gave cultures of *aerogenes* only while the other gave *Bact. coli* I, it seems likely, therefore, that this organism was present in the sample in minimal numbers and only attained prominence in the 37° C. tube by overgrowing the other type present. Upon this sample, a 44° C. faecal *coli* count was obtained also by subculture from the 37° C. positive tubes (Wilson's method IV), and as this showed a count of 1 only the above explanation is corroborated.

But other factors may be at work in such cases. It will be remembered that Harold (1935) showed that in order to give a positive result at 44·5° C.,

Table 5. *Direct inoculation* 44° C. *versus* 37° C. *with plating and differentiation. Presuming presence of* Bact. coli *I only when actually isolated*

Bact. coli type I:	
Undetected by either method	36
Detected by both methods	43
Detected at 44° C., undetected at 37° C. ...	13
Detected at 37° C., undetected at 44° C. ...	12
Samples examined	104

Table 6. *Presuming presence of* Bact. coli *I upon other evidence in three additional* 44° C.*-positive tubes*

Bact coli type I:	
Undetected by either method	36
Dectected by both methods	46
Detected at 44° C., undetected at 37° C. ...	13
Detected at 37° C., undetected at 44° C. ...	9
Samples examined	104

the original inoculum had to contain more organisms than were needed to achieve the same purpose at 37° C.; so it is possible that from samples like these which contain comparatively few organisms, the necessary number may not be forthcoming.

Another explanation which suggests itself is that strains of *Bact. coli* may exist which are capable of growing at 44° C. only after primary enrichment or after what might be described as a few hours of 'primary acclimatization' at 37° C.

The work of Skinner & Brown (1934) and of Minkevich, Alexandrov & Soboleva (1936) lend colour to this explanation, and Hajna & Perry (1939) deliberately use incubators in preference to water-baths in order to secure the time lag before full temperature is reached.

The strains of *Bact. coli* in the nine samples under consideration grew readily enough when subcultured into MacConkey broth at 44° C. after they

had been isolated from the 37° C. tubes; that is to say, when they had first undergone enrichment.

As to the thirteen samples in which 37° C. incubation and plating failed to detect *Bact. coli* type I when it was detected at 44° C.; it is a striking fact that in one sample only did the direct 44° C. count exceed 5 faecal *coli* per 100 ml. It must be assumed that when *Bact. coli* I is present in such small numbers, it can be so overgrown by other types in MacConkey broth at 37° C. that its chances of isolation become non-existent. Alternatively, the suggestion arises that, contrary to the ideas of some workers, a small inoculum is at least as well able to initiate growth at 44 as at 37° C. In nine of these samples the 37° C. count was never less than seven times and was in one case at least 180 times as great as the 44° C. count, but in only one sample was the count at 37° C. less than that at 44° C.

It is interesting that in Bardsley's (1938) much larger series *Bact. coli* I was missed in thirty samples by the 37° C. presumptive test with plating, and that in twenty-three of these less than 5 *Bact. coli* per 100 ml. were present—the same figure as that obtained in the present work. Bardsley concluded that when *Bact. coli* I was present in small numbers in relation to I.A.C. it was liable to be missed by this method, and it has been shown that this condition obtained in nine of the thirteen samples under consideration.

The question of overgrowth of one type by another in culture has evidently a great deal of bearing on the results obtained by plating and differentiation. Bardsley (1938) held the view that in MacConkey broth, *Bact. coli* I as a general rule overgrew I.A.C. organisms. Her opinion was not universally shared, for Harold (1936) and Mackenzie & Hilton-Sergeant (1938) took the view that the opposite might occur, while Perry (1939) went so far as to say that the Eijkman test had its greatest value in samples with a high content of *aerobacter* strains because these were apt to overgrow *Bact. coli* at 37° C. The truth is likely to be that the relative numbers of *Bact. coli* and I.A.C. in the original inoculum decide which type shall eventually gain ascendancy in the culture.

Out of sixty-eight samples which most probably contained *Bact. coli* I the direct 44° C. method detected this organism in thirteen (19%) in which it was undetected at 37° C., in spite of the fact that more colonies were being picked from the plates of the 37° C. series than would usually be taken in routine work.

On the other hand, in nine (13%) of the samples, the 37° C. method was successful at the expense of the 44° C. method.

The results of this comparison show that the direct 44° C. test is superior to the plating method for the detection of *Bact. coli* in water samples, and though that superiority is not very great numerically, it is in the type of sample in which I.A.C. greatly outnumber *Bact. coli* that it is especially valuable.

Note on irregular type I

In the present investigation thirty-four cultures appeared on first isolation to belong to this type. On repetition of the 44° C. MacConkey test, no less

than fifteen of these confirmed as 44° C.-positive and were transferred to the total of *Bact. coli* I. Such a prevalent shift of reaction demands some explanation and there are several possibilities:

(1) That these are unstable strains of *Bact. coli* I which can lose and regain the power of fermenting lactose at 44° C.

(2) That these are strains of *Bact. coli* I which need an unusually large inoculum in order to grow at 44° C. This does not appear likely in the present work, for the same loop was used both for the original inoculations and the repeats.

(3) That there are two types of Irregular I. One which ferments cellobiose and is usually 44° C.-negative; and another type which does not ferment cellobiose and is in fact a type of *Bact. coli* which is unstable in regard to fermentation of lactose at 44° C.

Though the present series of cultures was a small one an interesting fact was noticed. Leaving out of account six non-lactose-fermenters, twenty-one of the cultures classed as Irregular I on first isolation failed to ferment cellobiose, and of these fourteen confirmed as 44° C.-positive. On the other hand, out of seven cultures which fermented cellobiose, one only confirmed as 44° C.-positive.

It would be profitable to study a large series of Irregular I with the addition of cellobiose to the differential tests, and using very heavy inocula by pipette for the 44° C. MacConkey tubes to ascertain how many of the strains would then remain 44° C.-negative. It is interesting that Clegg (1941) encountered a large number of these 'shifting' strains even though he used a triple loop.

Results of cellobiose fermentation

Cellobiose was found inferior to citrate as an aid to identification of the I.A.C. group and 10% of cultures of *Bact. coli* type I were found to ferment it (Batty-Smith, 1942).

Results of Voges-Proskauer test

Barritt's test showed a close agreement with O'Meara's test, and it was found that more positive results were obtained by testing the cultures after 3 days' incubation than after 2 days (Batty-Smith, 1941).

PART III. GENERAL DISCUSSION

It is proposed to consider the Eijkman test under several headings in order to arrive at an estimate of present opinion upon its value. Since the specificity of the test depends so much upon the composition of the medium used, upon the exact temperature of incubation, and upon details of technique, it is clearly impossible to take into consideration work performed with higher temperatures than are now advocated or with the multitude of media that have been from time to time proposed. This, from the point of view of English readers, practically means considering only work performed using MacConkey broth at a temperature of 44° C. and since the publication of the work of Wilson *et al.* (1935).

A. *Specificity*

The question at issue here is whether any great proportion of I.A.C. strains can ferment lactose at 44° C., and the findings of various workers are collected in Table 7. The figures have been abstracted from the papers concerned by adding together the numbers of intermediate, *aerogenes* and *cloacae* strains. Two citrate-positive strains of *Bact. coli* I found by Clegg & Sherwood were not considered for the purposes of this table.

Table 7 shows that the most adverse comment on the specificity of the test in this country is contained in Taylor's results. The influence of this percentage of error in the selectivity of the test for *Bact. coli* I will clearly be dependent upon the proportion of samples in which I.A.C. are present apart

Table 7. 44° C.-*positive* I.A.C. *strains*

	No. of strains	% 44° C.-positive
Wilson *et al.* (1935)	266	0·4
Bardsley (1938)	1086	0·6
Clegg & Sherwood (1939)	324	0·6
Ferramola & Monteverde (1939)	169	1·1
Hajna & Perry (1939)	229	15·7
Taylor (1941)	76	9·2
Batty-Smith (this paper)	131	3·8

Table 8. *Percentage of* 44° C.-*positive tubes confirmed for* Bact. coli *I and Irregular II*

	No. of tubes	% proved *Bact. coli*
Met. Water Board (1937)	165	100·0
Met. Water Board (1938)	221	98·6
Mackenzie & Hilton-Sergeant (1938)	—	100·0
Bardsley (1938)	180	100·0
Ferramola *et al.* (1939)	—	93·0
Raven *et al.* (1940)	134	99·25
Ferramola (1940)	309	95·0
Clegg (1941)	1766	97·6

from *Bact. coli* I in any given series. Taking Bardsley's series of 550 samples as a representative one, 349 contained both *Bact. coli* and I.A.C., and the practical effect of the 9·2 % error would be reduced in such a series to negligible proportions. In any case, the large proportion of 44° C.-positive I.A.C. found by Taylor as compared with other workers argues that the distribution of such types is localized, and Raven *et al.* (1940) and Harding (1940) have expressed this opinion. The higher figure obtained by Hajna & Perry (1939) may also have a local significance (for their work was performed using MacConkey broth at 44° C.) though the writer feels that their employment of an incubator instead of a water-bath may be a factor here.

Results are given by other workers in a form which makes them difficult to tabulate and among these should be mentioned: Raven *et al.* (1940) who isolated two 44° C.-positive *aerogenes* strains from 134 tubes examined and Clegg (1941) who encountered a single 44° C.-positive *aerogenes* among what must have been a very large number of strains.

A very different state of affairs is found by workers in India. Raghavachari & Iyer (1940) found that about 75% of their *aerogenes* I strains were 44° C.-positive, and Banerjea & Sen (1940) about 45%. These results need not concern users of the method in this country, but if—and evidence to this effect has been produced by these workers—it is confirmed that these 44° C.-positive *aerogenes* strains are true faecal organisms in the tropics, it will not invalidate the usefulness of the test in those regions.

In Table 8 specificity is considered from a different angle (the percentage of 44° C. primary positive tubes confirmed for *Bact. coli* I) and is shown to be uniformly high, the only figures below 98% being those of Ferramola and his colleagues which were obtained from waters in the Argentine. Comment on so high an efficiency is superfluous.

B. *Relative efficiency for the detection of* Bact. coli *I of methods I and IV (Wilson)*

By method I is understood the 37° C. presumptive coliform test with plating and differentiation.

Table 9 shows that by Bardsley's and the Metropolitan Water Board's (1938) results, method IV shows an improvement of about 5% upon method I, while the figures of Raven *et al.* show method IV at a disadvantage by about 3%. It is significant that, in their series, among the fifty-three samples in

Table 9. *Relative efficiency for detection of* Bact. coli *and Irregular II of methods I and IV*

	No. of samples	*Bact. coli* detected by		*Bact. coli* missed by	
		Method I	Method IV	Method I	Method IV
Bardsley (1938): Water	550	417	446	30	1
Faeces	100	96	100	—	—
Raven *et al.* (1940)	329	113	104	22	31
	No. of + tubes				
Met. Water Board (1938)	1223	950	1018	—	—
Met. Water Board (1937)	186	167	165	—	—

which *Bact. coli* was missed by one method or the other no count higher than 5 per 100 ml. was recorded either at 37 or 44° C.; the sampling error was therefore probably responsible for these discrepancies. There appears to be a general consensus of opinion that method IV detects about the same or a slightly greater number of *Bact. coli* than method I. Even if its efficiency were slightly less than that shown by method I, it would still be well worth employing on account of its superior speed, economy and simplicity as compared with plating and differentiation.

C. *Direct inoculation of water with incubation at* 44° C. *(Wilson's method III) compared with methods I and IV*

Very little work has been done with method III which is not reported on so favourably as method IV. The Metropolitan Water Board (1937) found it less effective than method I, but the number of samples tested was very small.

In 1938 the Board again investigated method III upon 621 samples with results so inferior, as regards recovery of *Bact. coli*, to their 37 and 42° C. presumptive tests that they dismissed the method forthwith, the number of *Bact. coli* recovered having been only 69% of that at 37° C.

Mackenzie & Hilton-Sergeant (1938) found that this method detected the presence of *Bact. coli* when it was missed by the 37° C. presumptive test. The writer's own results as compared with 37° C. and plating have already been given in this paper (p. 84) and show a definite superiority to method I.

The only other comparison of any extent between methods III and IV has been that of Clegg (1941), whose remarks are considered in detail on p. 75. His results agreed fairly closely with those of the M.W.B. (1938), and it would be difficult to improve upon his summing up of the case for and against method III when he says that although this method is inferior to method IV as regards recovery of *Bact. coli* it can nevertheless fill a useful place on account of the speed with which a result can be obtained.

Table 10. *Increase of positives during second* 24 *hr. of incubation at* 44° C.

	Increase in positives during 2nd 24 hr.
Wilson *et al.* (1935)	10%
Met. Water Board (1938)	1·4%
Mackenzie & Hilton-Sergeant (1938)	10%
Report (1939)	Some strains need 72 hr.
Clegg & Sherwood (1939)	Few
Raven *et al.* (1940)	Few
Ferramola (1940)	5%

D. *Period of incubation at* 44° C.

Most workers advocate 48 hr. incubation for achieving the full number of positive results, but some have found 24 hr. satisfactory and others have recorded the precise increase of positives during the second 24 hr. Various opinions and remarks are shown in Table 10.

It is evident that the shorter period is adequate for routine testing especially in waterworks practice or in emergencies, but that 48 hr. should be given if possible. Minkevich, Alexandrov & Soboleva (1936) found that the maximum amount of gas was produced during the first 24 hr., hardly increasing at all during the second 24 hr. and that nearly the maximum production had been reached after 16 hr. It should be noted that this work was performed using Bulir's mannitol broth at 43·5° C., but it is nevertheless significant. In the writer's opinion a useful report could be made after 16–18 hr. in circumstances of exceptional urgency.

E. *Origin and frequency of Irregular type II*

Since this organism is 44° C.-positive it is important to discover whether it is a true inhabitant of the human intestine and whether it is frequently present in water samples.

Regarding its presence in faeces, Dr Windle Taylor of the Metropolitan Water Board tells me that he found 3·3% of this type in 276 samples of human faeces and a considerable number in water samples. He therefore inclines to think it should not be dismissed too lightly.

Bardsley (1938) quoted Wilson *et al.* (1935) as reporting the presence of 3·2% of Irregular II among 125 strains isolated from fifty samples of cow dung, but she herself found none in 100 samples of human faeces.

Ferramola & Monteverde (1939) found that 'typical faecal *Bact. coli* type II (i.e. unable to produce indole)' formed 1·23% of coliform bacteria isolated from Buenos Aires sewage; the perusal of other papers by Ferramola suggests that Irregular II is the organism they mean.

Bardsley (1938) found Irregular II in eight samples of water, in five of which it was associated with *Bact. coli* I. She found it difficult to express an opinion on its habitat, but considered that its frequent association with *coli* I opened it to grave suspicion. It was in 0·5% of samples only that its presence caused over-estimation of the faecal *coli* count. Mackenzie & Hilton-Sergeant (1938) found 1·8% of Irregular II among 496 strains from faeces.

Harold (1937) found that of 165 positive 44° C. MacConkey broth tubes obtained by subculture from 37° C. positives, seven or 4% contained '*Bact. coli* II' by which Irregular II is presumably meant.

Ferramola (1940) in a series of 309 44° C. positive tubes obtained from water samples, found three or 1% confirmed for '*Bact. coli* II'. From sewage, among 222 strains he found only one strain, and it was absent from most samples of faeces from birds and cattle.

Irregular II was isolated from two samples out of the 104 in the writer's series, and in each case the sample contained *coli* I.

The percentage of Irregular II found in faeces does not make it certain that it is a true faecal organism, and in any case its prevalence in water samples is not high enough to interfere seriously with the 44° C. MacConkey test, especially as there is some suspicion of its being a faecal organism.

F. *False reactions in MacConkey broth at* 44° C.

These may be (1) Real:

- (*a*) due to Gram-positive spore-bearers,
- (*b*) due to 44° C.-positive I.A.C. organisms.

(2) Apparent:

- (*a*) due to *Bact. coli* I which has died out,
- (*b*) due to *Bact. coli* I which has lost its power of fermentation on subculture and is in consequence identified on plating as Irregular I.

No figures are available as to prevalence of false positives due to Gram-positive organisms, but since, according to Wilson & Blair (1925) there is in

practice a fair degree of correlation between the *Bact. coli* and sulphite-reduction tests, and since the presence of *Cl. Welchii* may indicate serious pollution of an intermittent kind, 'false' positives caused by these organisms would seem to be of academic interest rather than of practical importance.

The causes of the other three types of false positive have already been discussed.

G. *Methods III and IV—technical considerations*

Besides the differences in the actual numerical results, each method has its advantages and disadvantages for routine use; these may be set forth as follows:

By the direct method it is possible to report the result after 48 hr., or even after 24 hr. in urgent cases; whereas until the 37° C. tubes have had their 48 hr. incubation, the seeding method cannot even be begun and so cannot be reported until the fourth (or in urgent cases the third day) after receipt of the sample. Rapid results are so universally desired—particularly by waterworks engineers—that this advantage of the direct method is important.

It is a disadvantage of the direct method that owing to the use of double strength medium and of its large 50 c.c. tubes, it is more expensive in materials; also that the larger number of tubes and their greater size necessitate the provision of larger water-baths.

If the seeding method is used, only small test-tubes containing 5 c.c. of single-strength broth are needed and fewer tubes are wanted, for only when examining highly polluted samples will a complete set have to be inoculated.

In favour of the direct method it may be said that to pipette water into tubes is a quicker and easier operation than to inoculate them from other tubes with a wire loop, though the writer admits that this may be a personal preference.

Mention may be made here of a small matter connected with Durham's tubes. The *Report* (1939) recommends $3 \times \frac{7}{16}$ in. gas tubes for the 50 ml. tubes, and such tubes have a capacity of about 10 ml. In order to keep down the fluid level in the 50 and 10 ml. tubes and so prevent wetting of the plugs, it is the custom of some workers to use 25 and 5 ml. respectively of *triple* strength MacConkey broth instead of 50 and 10 ml. of double strength. The writer has noticed that, on inoculation, the contents of the gas tube do not diffuse out into the bulk of the fluid even in 48 hr. unless gas is formed to push them out. This means that 50 ml. of water are in effect inoculated into only 15 c.c. of triple-strength broth which must seriously reduce the final concentration of the constituents of the medium. The same thing applies in a lesser degree when double-strength broth is used and in a similar way in the 10 ml. tubes. It is the custom at the Metropolitan Water Board's laboratories to use for the 50 ml. tubes a short gas tube of approximately the usual diameter ut only 2 in. in length. This seems an effective way of combating this difficulty.

H. *Conclusions*

There is to-day a great body of evidence in support of the 44° C. MacConkey test. In this paper an endeavour has been made to demonstrate, from the work of others and from a small amount of original investigation, the reliability of the test and to indicate how it may best be used in the routine bacteriological examination of water samples.

In the writer's opinion it can be justly described as the finest single test for the detection of *Bact. coli*, and it is not too much to say that the practice of any laboratory engaged in water analysis is incomplete without this test, for it has been shown in these pages that not only is it a more sensitive method than the old method but that it is much more economical in materials, time and labour.

The time has therefore come when standards should be laid down in order that the information which the method gives shall be backed by the weight of authority. Waterworks engineers, Medical Officers of Health and Sanitary Inspectors alike demand rigid standards, for such are a sure answer to criticism or complaint. If any laboratory were to begin to send out reports of the presumptive faecal *coli* count at the present time it would almost certainly meet with two difficulties.

The first of these would consist of requests for definite rulings upon the results: 'Is this sample satisfactory or unsatisfactory?' and at the present time no definite answer can be given, for the application of the current Ministry of Health standards to the faecal *coli* count could only lead to chaos.

This is not the place to enter the never-ending controversy which rages round the relative significance of the various members of the coliform group in relation to the sanitary quality of waters. Although the position of the *Bact. coli* as the indicator par excellence of pollution has been assailed from time to time, it is still supreme, but the majority of water bacteriologists now agree that the presence of organisms of the intermediate-*aerogenes-cloacae* group and even of spore-bearers and of symbiotic glucose fermenters, can give information of equal—though of different—importance.

That being so, it is unlikely that the faecal *coli* count can supplant the presumptive coliform count; the two must be worked in conjunction. It is generally agreed that the *Bact. coli* is an indicator of present pollution and the I.A.C. group of remote or of imminent pollution. Two sets of standards should be laid down; one to indicate the allowable number of faecal *Bact. coli* per 100 ml., the other, rather more lenient, for the number of I.A.C. organisms and samples should have to comply with both sets.

The second difficulty arises out of the method of reporting. When using method III it is easily possible, owing to the sampling error of the method, for the presumptive faecal *coli* count to be higher than the presumptive coliform count, and the recipient of the report justifiably asks how it is that the number of faecal *coli* can be higher than the total number of coliforms.

It is surprising how sceptically the explanation is received even by medical officers and waterworks engineers, and this tends to discredit the method.

Wilson *et al.* (1935) suggest a sound method for dealing with this difficulty. They say: 'The number of positive tubes at 37° C. or 44° C., whichever is the higher, is used for estimating the presumptive coliform count, the number of positive tubes at 44° C. for estimating the faecal *coli* count,...', and this method of reporting should be officially recognized.

A differential medium which can detect the I.A.C. group without giving positive results with atypicals is still much to be desired, and it has here been shown that cellobiose does not fill the gap with any great distinction.

Wilson's methods nos. III and IV were designed to provide, by means of 44° C. incubation and of inoculation of Koser's citrate from the 37° C.-positive tubes, three presumptive counts: (1) the presumptive coliform count, (2) the faecal *coli* count and, (3) the I.A.C. count. Unfortunately, the success of this scheme depended upon the specificity of citrate for the I.A.C. group and no. 3 was a partial failure.

Cellobiose could only be worse than citrate here, for it gives as many positives with atypicals as does citrate, and in addition a considerable number of faecal *Bact. coli* can ferment it. The work of Stuart *et al.* (1940) upon the slow lactose-fermenting coliforms has already been noticed in this paper, and if their conclusions and their tentative classification are found to represent a real advance, we may have to widen our present conception of the coliform group to include many organisms which we now loosely describe as 'non-lactose-fermenters'.

Summary

1. A historical survey is given of work upon the Eijkman method.

2. A total of 104 water samples were examined by methods I and III. These yielded 602 cultures obtained from the 44 and 37° C. presumptive series, and of these 357 were 44° C.-positive. The only types capable of fermenting lactose at 44° C. were *Bact. coli* type I and Irregular type II, with the addition of three cultures of intermediate type I and two of *aerogenes* type I.

3. The relative efficiencies in the detection of *Bact. coli* of the 44° C. method by direct inoculation and of the 37° C. presumptive test with plating and differentiation were compared. Out of 104 samples, sixty-eight were shown to contain faecal *Bact. coli*, and in 19% of these the 44° C. method detected *Bact. coli* when it was not recovered at 37° C. The converse was true in only 13% of samples.

4. The results obtained by other workers in regard to the specificity of incubation at 44° C. and to the various methods hitherto proposed for employing it have been collected and discussed; technical advantages and disadvantages have also been considered.

5. It is concluded: (1) That false positives at 44° C. are a negligible quantity for practical purposes. (2) That the best all-round method to use is Wilson's method IV. (3) That 48 hr. incubation should be given, but that 24 hr. is sufficient when rapidity is desired. (4) That the test should be officially recognized and standards laid down for use with it.

6. Cellobiose was found to be inferior to citrate for the identification of the I.A.C. group.

My thanks are due to Prof. P. L. Sutherland, Director of this laboratory, for permission to publish these results; to Dr E. Windle Taylor, Senior and Research Assistant, Metropolitan Water Board, for details of his own experiments, for the loan of certain journals and for advice and criticism; and to Mr A. Lonsdale, Senior Laboratory Assistant, for extra work involved in inoculating water samples.

Table 1 on p. 62 is reproduced from the *Spec. Rep. Series Med. Res. Council*, no. 206, by permission of the Medical Research Council and the Controller of H.M. Stationery Office.

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(*MS. received for publication* 28. XI. 41.—Ed.)