AN INTERLABORATORY COMPARISON OF RAPID METHODS FOR CONFIRMING THE IDENTITY OF COLIFORM ORGANISMS

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AN INTERLABORATORY COMPARISON OF RAPID METHODS FOR CONFIRMING THE IDENTITY OF COLIFORM ORGANISMS

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RESTRICTION: Issue only to Strathclyde Regional Council and WRc staff associated with the project and DoE nominated officers

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I OBJECTIVES

To ascertain if tests for β-galactosidase and β-glucuronidase can be used as alternatives to existing standard methods for confirming the identity of coliform organisms or E.coli.

II REASONS

A survey of water industry microbiologists carried out by the Rapid Methods Panel of the Standing Committee of Analysts Working Group 2 has shown that there is a real need for coliform confirmation techniques which are more rapid than those currently recommended.

III CONCLUSIONS

1. The β-galactosidase test would appear to be a suitable means of determining rapidly whether an organism has the potential to produce acid from lactose. However, since it gives no information about gas production, the β-galactosidase test does little more than confirm that colonies identified as lactose fermenters on primary isolation media are indeed able to utilise this carbohydrate. The value of using this test instead of assuming that all lactose fermenting colonies on selective coliform media are in fact coliform organisms is therefore questionable.

2. If it is agreed that use of the β-galactosidase test has some merit, the RAPIDEC method would appear to be the most efficient in that it gives results in 2 hours with only a 1 per cent chance of failure to alert.
3. On the basis of the data obtained in this trial, the β-glucuronidase test would appear to be less specific for *E coli* than has been previously reported. However, this may be due to the use of LTMB medium, rather than a more systematic scheme, as the reference method for identifying *E coli*. In comparison to LTMB, the most sensitive β-glucuronidase test, RAPIDEC, failed to identify 11 per cent of the isolates as *E coli*. Of the isolates not identified as *E coli* by LTMB, 22 per cent possessed the β-glucuronidase enzyme.

4. On the basis of these results the LTMB and β-glucuronidase tests cannot be regarded as equivalent although the rapidity of the β-glucuronidase test may make it a useful additional test in certain operational situations.

IV RECOMMENDATIONS

Recommendations will be made once the results of this study have been discussed by the Rapid Methods Panel of the Standing Committee of Analysts Working Group 2.

V RESUME OF CONTENTS

The results of a trial to compare the validity of using β-galactosidase and β-glucuronidase tests for confirming the identity of coliform organisms are given. In the trial several commercially available forms of the β-galactosidase and β-glucuronidase tests were compared with the existing recommended coliform confirmation procedures. A new method developed by Strathclyde Regional Council for detecting indole production and β-galactosidase activity was also included. The trial was organised by Strathclyde Regional Council with
guidance from the Rapid Methods Panel of the joint DoE/PHLS Standing Committee of Analysts Working Group 2. The study, which involved eleven water microbiology laboratories, was part-funded by the Department of the Environment.
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1. INTRODUCTION

In microbiological water quality monitoring, the most frequently used parameters are the faecal indicator bacteria (coliform organisms and *Escherichia coli*). In the UK, water undertakings annually carry out more than 1.5 million determinations.

Water undertakings currently follow the procedures outlined in Report 71 (*DoE et al* 1983) when isolating and identifying coliforms and *E. coli*. The techniques in Report 71 are currently being revised and updated by the joint DoE/PHLS Standing Committee of Analysts Working Group 2 on the microbiological examination of water, sewage etc. A separate Panel has been established to consider the progress with rapid methods.

A survey of Water Industry Microbiologists carried out by the Rapid Methods Panel of Working Group 2 has shown that there is a demand for a reliable, rapid method for confirming the identity of coliform and *E. coli* isolates. There is also a demand for rapid bacteriological techniques which do not involve the use of expensive or large capacity instruments.

UK practice for the confirmation of coliforms and *E. coli* is based on the ability of these organisms to produce gas, from the fermentation of lactose at 37 °C for coliforms, or from the fermentation of lactose or mannitol at 44 °C for *E. coli*. In addition, for *E. coli* the ability to produce indole from tryptophan at 44 °C must be demonstrated. It is essential to carry out these confirmation tests when potable water supplies are being examined. Using existing methods, a delay of up to 24 hours
can occur between obtaining the presumptive and confirmed detections of coliforms or *E. coli*. This may delay the instigation of appropriate investigations, or decisions on remedial action.

Water Undertakings often take action on the results of presumptive detections of coliforms and *E. coli*. However, if the presumptive isolates do not confirm, the expenditure incurred in remedial action will have been wasted, and if this occurs repeatedly, water quality managers will lose confidence in microbiological monitoring. On the other hand, postponing action until the identity of isolates is confirmed may put the consumer at risk.

There is a generally recognised need for methods that will detect coliforms and *E. coli* rapidly, with the reliability and sensitivity of the currently recommended tests.

Many workers have developed and described single-tube confirmatory tests for *E. coli* (Schubert 1956, Fennell 1972, Pugsley *et al* 1973), and lauryl tryptose mannitol broth (LTMB) with added tryptophan was adopted as a recommended test in the UK in 1983 (*DoE et al* 1983). With LTMB and related media, mannitol is used as the fermentable carbohydrate instead of lactose. Fennell (1972) demonstrated that use of a medium containing mannitol instead of lactose reduced the number of false negative results caused by strains of *E. coli* which were unable to ferment lactose, or which did so very slowly.

Taylor (1955) has shown that it is possible to detect gas and indole formation with single tube media after incubation for only 6 hours at 44 °C, but tests should not be regarded as negative until
after 24 hours incubation. The mannitol media used in single tube confirmatory tests can also be used for the confirmation of coliforms, by demonstrating production of gas at 37 °C.

Enzymic tests can be rapid, sensitive and specific, and β-galactosidase and β-glucuronidase have been suggested as replacements for the traditional confirmatory tests which involve detecting acid and gas production from lactose. The fermentation of lactose by Enterobacteriaceae involves a permease enzyme, which enables the lactose to enter the cell, and a β-galactosidase enzyme which attacks the β-galactoside link in lactose, hydrolysing it to glucose and galactose. An organism may fail to ferment lactose if it lacks either the permease or the β-galactosidase. The presence of β-galactosidase in the absence of the permease can be demonstrated by using ortho-nitrophenyl-β-D-galactoside (ONPG) as the substrate. This colourless substance is attacked by the β-galactosidase causing the release of yellow o-nitrophenol (Lapage and Jayaraman 1964).

One of the rapid single tube media (RST) assessed in this trial uses ONPG as the substrate. RST medium was designed to permit the most rapid growth of low numbers of E. coli, and to produce the optimum indole response when compared with LTMB and the multiple tube medium, minerals modified glutamate (MMG) (Taylor unpublished). In RST medium, the concentration of ONPG was adjusted to give the best balance of β-galactosidase and tryptophanase activity after the minimal growth periods of 6-12 hours.

β-glucuronidase is an enzyme which is thought to occur, among the Enterobacteriaceae, only in E. coli, and in some salmonellas and shigellas.
Kilian and Bulow (1976) found that approximately 97% of *E coli* strains produced β-glucuronidase. This enzyme can be detected in about 4 hours in broth cultures or from isolated colonies.

The purpose of this study was to undertake a trial of rapid confirmation procedures for coliforms and *E coli* using enzymic tests, and to ascertain whether the tests could be used as an alternative to the procedures laid down in Report 71 (DoE et al 1983). The study was designed to collate and examine the data from 1100 coliform cultures isolated and tested in 11 participating laboratories. Several forms of both the β-galactosidase and the β-glucuronidase test were evaluated.

The study was carried out using several commercially-available forms of the tests, and RST medium produced by Strathclyde Regional Council. The performance of these was compared with that of the commercially-available formulation of LTMB (DoE et al 1983).

2. ORGANISATION OF THE TRIAL

A total of 11 laboratories participated in the trial. Water Authorities in England and Wales were represented by laboratories from Anglian, North-West, Severn-Trent, Thames, Yorkshire, Welsh and Wessex. Water Companies were represented by Eastbourne Water Works Company, and Scottish Regional Councils by Lothian and Strathclyde. The WRc Medmenham Laboratory also participated in the trial, which was designed to allow each of the 11 laboratories to examine 100 environmental isolates. The trial was funded by the Department of the Environment, who contracted Strathclyde Regional Council Water Department to carry out the following tasks:
a. Manufacture sufficient ampoules of RST medium.
b. Order sufficient chosen commercial test kits.
c. Design the trial and produce a detailed protocol.
d. Collate the results.
e. Produce a report for DoE.

A total of £2500 was allocated to this project, with £1800 to materials and £700 to staff costs. In late December 1987 a draft protocol, results sheets (Appendix A) and 80 grams of commercially-produced LTMB (Oxoid CM 832, Lot No 275 38755) were sent to each of the trial participants.

In early January 1988 a meeting of the Rapid Methods Panel of SCA/PHLS Working Group 2 was called to discuss the design, protocol and organisation of the trial. Participants from Strathclyde Water Department, Severn-Trent, Thames, Wessex and Yorkshire Water Authorities, DoE and WRc were present. It was decided that chlorinated waters were the preferred sources of isolates, but due to the time scale of the project, if necessary isolates from surface waters could also be used. Around 50 isolates would be taken from membranes incubated at 37 °C (presumptive coliforms), and 50 from membranes incubated at 44 °C (thermotolerant coliforms). A collaborative decision was reached on which manufacturers' test kits should be included in the trial, and this represented a balance between completeness and a feasible number which could be achieved. The logistics of carrying out the trial, and the provision of sufficient
culture for simultaneous inoculation of several tests, were also discussed. The views of the meeting were incorporated into the final protocol.

In late January 1988 all the media required for the trial, finalised protocol, detailed instructions and results sheets were sent to all participating laboratories. The time taken to complete the trial was anticipated to be around 4-6 weeks.

An application to DoE to extend the date of completion of the trial, and to subcontract a portion of the work to WRc was submitted in March 1988. Additional funding of £2250 was granted by DoE to Strathclyde in April 1988, and a subcontract was set up with WRc Medmenham to collate the results obtained from the 1015 isolates examined. An additional extension to the completion date of 31 March 1988 was granted by DoE.

3. DESIGN OF THE TRIAL

Participants were asked to start collecting isolates of presumptive coliforms and \textit{E. coli} after they received their commercially produced LTWB. At the same time, isolates were confirmed using the participating laboratories' usual confirmatory medium. Around 100 colonies were collected by each laboratory with as many colonies as possible from final (ex-works) chlorinated waters. Where possible, the remaining colonies were collected from a cross-section of different types of raw waters. Each laboratory selected both large and small colonies, 50 from 37 °C membranes, and 50 from 44 °C membranes. Colonies were then checked by the oxidase test, and all colonies which were oxidase positive were discarded. Oxidase negative colonies were inoculated into LTWB, the
participating laboratories' usual confirmatory medium, and to a plate or slope of nutrient agar plate for testing at a later date.

Colonies from the 37 °C and 44 °C membranes were confirmed at both 37 °C and 44 °C in LTMB and in the laboratories' usual confirmatory medium. The results of growth, gas and indole production were recorded after 6 hours and 24 hours.

The remainder of the analyses were carried out using inocula taken from the nutrient agar plate. Other tests were carried out using RST medium, API RAPIDEC test kits, β-galactosidase tablets (Lab-M and Oxoid) and β-glucuronidase tablets (Lab-M and Mast). Results were recorded after the times shown in Table 1, and all tests to be read after 6 hours were completed within the working day.

Table 1. Incubation times for rapid confirmation tests

<table>
<thead>
<tr>
<th>Tests from nutrient agar plate</th>
<th>Results recorded after</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. LTMB medium at 37 °C and 44 °C</td>
<td>6 h and 24 h</td>
</tr>
<tr>
<td>b. RST medium at 37 °C and 44 °C</td>
<td>2 h, 6 h and 24 h</td>
</tr>
<tr>
<td>c. RAPIDEC coli</td>
<td>2 h and 6 h</td>
</tr>
<tr>
<td>d. Lab-M β-galactosidase and β-glucuronidase tablets</td>
<td>2 h and 6 h</td>
</tr>
<tr>
<td>e. Mast β-glucuronidase tablets</td>
<td>2 h and 6 h</td>
</tr>
<tr>
<td>f. Oxoid β-galactosidase discs</td>
<td>2 h and 6 h</td>
</tr>
<tr>
<td>g. API RAPID 20E</td>
<td>4 h</td>
</tr>
</tbody>
</table>

The methods for carrying out a-g are outlined in Section 4. Strains which gave anomalous results in API RAPID 20E were further identified with API 20E, or were kept on nutrient agar slopes. Results were recorded on standard forms, and these were returned to WRc Medmenham for further analysis.
4. TEST PROCEDURES

4.1 LTMB medium

Commercially-produced LTMB (Oxoid CM831, Lot number 275 38755) was used by the 11 participating laboratories. The medium was prepared according to the manufacturer’s instructions, which were as follows: Suspend 35.8 g of LTMB in 1 litre of distilled water, and heat gently to dissolve. Distribute into final containers in 5 ml amounts and insert an inverted Durham tube. Sterilise by autoclaving at 115 °C for 10 minutes. The final pH should be 6.8 +/- 0.2.

Test procedure: Two tubes of LTMB were inoculated from each nutrient agar plate, one was incubated at 37 °C, and the other at 44 °C. The results of both tests were read after 6 and 24 hours. The indole test was carried out after 6 hours by adding 0.025 ml of Kovacs reagent to 0.5 ml of culture removed from each tube. After 24 hours, 0.025 ml of Kovacs reagent were added to the LTMB tube.

4.2 RST medium

RST medium was prepared to the formula of Taylor (1982). It was designed from lauryl tryptose lactose broth (Oxoid CM451) because it permitted the most rapid growth of low numbers of E coli, and gave better indole reactions than LTMB, minerals modified glutamate medium (MMGM) or EC medium. It uses o-nitrophenyl-β-D galactoside (ONPG) as the substrate, and the amount incorporated into the medium was adjusted to give the best balance of β-galactosidase and tryptophanase activity after minimal growth periods (ie 6 to 12 hours).

Test procedure: Two or three medium-sized colonies were picked from the Nutrient Agar plate and subcultured to two tubes of RST medium. The
suspensions were thoroughly mixed and one tube was incubated at 37 °C, and the other at 44 °C. The results of β-galactosidase and indole tests were read after 2, 6 and 24 hours. A positive β-galactosidase reaction was denoted by the medium changing colour to golden yellow. After thoroughly mixing the RST medium, 0.5 ml volumes were removed from the 37 °C and 44 °C tubes after 2, 6 and 24 hours into clean tubes, and tested for indole production using Kovacs reagent.

4.3 RAPIDEC coli (API)

Commercially produced RAPIDEC coli is a standardised micromethod for the identification of Escherichia coli in 2 hours. Each RAPIDEC kit allowed 50 tests to be performed. Each kit consisted of 10 strips with 5 detections on each strip, 10 lids, and one instruction manual. Also included were sterile applicator sticks and JAMES reagent for the indole test.

Test procedure: Each individual test used 4 cupules on the strip, marked C, S, 1, and 2. Sterile distilled water (150 µl) was added to cupules C and S. The contents of cupule C were homogenised with a wooden applicator strip. This produced an turbidity standard equivalent to McFarland No 3.5. Using a different stick, colonies were emulsified in cupule S to give a turbidity similar to that of cupule C. Cupules 1 and 2 were each inoculated with 50 µl of the suspension from cupule S, the liquid was withdrawn from cupule C, and the strip was covered and incubated at 37 °C.

The strip was examined after 2 hours and 6 hours. Negative results were indicated by the contents of cupules 1 and 2 remaining colourless. A yellow
colour indicated a positive β-glucuronidase (cupule 1) or β-galactosidase (cupule 2) reaction. After incubation for 6 hours, a drop of JAMES reagent was added to cupule 2. The immediate formation of a pink colour indicated a positive indole-formation test.

4.4
β-galactosidase and β-glucuronidase tablets (Lab-M)

Five vials, each with 25 tablets of both commercially produced β-galactosidase and β-glucuronidase (Lab-M) were provided to each of the participating laboratories. The following items were also prepared: 200 clean tubes or vials, each with 0.25 ml of sterile physiological saline.

Test procedure: Two dense 'milky' suspensions (of at least MacFarland No 2 standard) were prepared by transferring colonies from each nutrient agar plate into two tubes containing 0.25 ml of sterile physiological saline. A β-galactosidase tablet was added to one tube, and a β-glucuronidase tablet to the other. Both tubes were incubated at 37 °C and examined after 2 and 6 hours. A positive result was denoted by a yellow colour for both tests.

4.5
β-glucuronidase discs (Mast)

Vials containing 125 commercially produced β-glucuronidase discs (Mast) were provided to each of the participating laboratories. The following items were also prepared: 100 clean tubes or vials, each containing 0.5 ml of sterile physiological saline.

Test procedure: A dense 'milky' suspension (of at least MacFarland No 4 standard) was prepared by transferring colonies from each nutrient agar plate into a tube containing 0.5 ml of sterile
physiological saline. A β-glucuronidase tablet was aseptically added, and each tube or vial closed. The tube or vial was incubated at 37 °C and examined after 2 and 6 hours. A positive result was denoted by a yellow colour.

4.6

β-galactosidase discs (Oxoid)

Vials containing 130 commercially produced β-galactosidase discs (Oxoid) were provided to each of the participating laboratories. The following items were also prepared: 100 clean tubes or vials and 20 ml of sterile physiological saline.

Test procedure: A disc was aseptically placed into each tube or vial, and 0.1 ml of sterile physiological saline was added. A loopful of the organisms under test was emulsified in the tube, which was then incubated at 37 °C and examined after 2 and 6 hours. A positive result was denoted by a yellow colour in the supernatant.

4.7

RAPID 20E (API)

Commercially produced RAPID 20E is a system which has been designed by API for the identification of Enterobacteriaceae in 4 hours after the microorganism has been isolated. It is a standardised system which consists of 20 biochemical tests. The system is made up of microtubes containing dehydrated substrates which demonstrate enzyme activity or carbohydrate fermentation.

The RAPID 20E kit allows 25 identifications, and consists of RAPID 20E strips, incubation trays, polyethylene pipettes, results sheets and one instruction manual. Also included were one bottle of mineral oil, one pack of reagents and one MacFarland 0.5 turbidity standard.
Test procedure: Colonies were emulsified in 1.25 ml of sterile 0.85% sodium chloride solution to give a turbidity equal to the McFarland 0.5 standard. This suspension was then used to inoculate the tubes of the RAPID 20E strip. Tubes which required anaerobic conditions (3 out of the 20) were sealed with mineral oil, and the strips were incubated at 37 °C for 4 hours. At the end of the incubation period, the spontaneous colour changes were recorded on a special coding form, and the necessary reagents were added to reveal the remaining test results. A 7-digit profile number was calculated from the results, and this was used with the Apilab computerised profile recognition system to obtain the strain identification.

4.8
API 20E

Commercially produced API 20E is a standardised identification system for Enterobacteriaceae and other Gram negative rods, which utilises 23 miniaturised tests and a database. The API 20E strip consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension which reconstitutes the media. During incubation, bacterial metabolism produces colour changes which are either spontaneous or revealed by the addition of reagents after 18 to 24 hours.

The API 20E kit allows 25 identifications and consists of API 20E strips, incubation boxes, report sheets and one instruction manual.

Test procedure: An incubating tray was prepared by placing 5 ml of water in its base to create a humid atmosphere. A 20E strip was then placed in the tray. A colony of the test organism was emulsified in 5 ml of sterile distilled water, and this
suspension was used to inoculate the tubes of the 20E strip. For most tests, only the tube part of each well was filled, but for three (indicated on the strip) the cup part was filled as well. Tests requiring anaerobic conditions (5 in all) were sealed with mineral oil. The cover was placed on the incubating tray, and the strip was incubated at 37 °C for 18 to 24 hours. After this period the strip was read by referring to the printed interpretation table, and the necessary reagents were added to develop the tests which did not give spontaneous colour reactions. A 7-digit profile number was calculated from the results, and this was used with the Apilab computerised profile recognition system to obtain the strain identification.

5. RESULTS AND DISCUSSION

5.1 Coding and entering of data

A separate data file was created for each laboratory taking part in the trial. The results of a single record containing a string of characters. The record included a number to identify the particular colony, and simple character codes for the type of sample, size and colour of the colony, and the temperature of incubation of the membrane from which the colony was initially picked.

The results of the individual tests carried out on each isolate were then entered as a string of single characters, "+" for positive results, "-" for negative ones, and "*" if the result was missing or recorded as doubtful. The test results were entered in a set order, so that each test was represented by a particular character position within the string. Also included was the 7-figure
profile obtained using API RAPID 20E, and a two-letter code giving the identification of the isolate, if one was successfully made. If a particular isolate was not identified by API RAPID 20E, but was subsequently satisfactorily identified by API 20E, the two-letter code reflected this identification, not the first, and an asterisk was inserted after the identification code to indicate this fact.

It soon became apparent that many inconsistent API RAPID 20E results had been recorded on the returned forms. This appeared to be because some laboratories had been using an obsolete profile index to obtain the strain identifications. To ensure consistency, the Apilab Profile Recognition System (running on an Apple IIe microcomputer) was used to re-assess all the profiles from laboratories which had not used this method when compiling their returns.

5.2 Validation of the data

Each set of results was entered twice, by a different operator on each occasion. Each pair of data files was then compared character by character, and any differences recorded. Reference was then made to the original results sheets, and the files were edited to correct any errors. The process of comparison was repeated until both files were identical, and the data were then assumed to be correct. This system of checking would not detect occasions when both operators had made identical mistakes, but the chances of this happening are relatively small. Overall, remarkably few errors were found, so the accuracy of the stored results is considered to be satisfactory. Initially the data were loaded on to
the Digital Vax system at the WRc Information Centre, but for later analysis the files were transferred to an Amstrad PC1640HD microcomputer.

5.3 Results obtained

Eleven laboratories took part in the trial. Each was asked to examine 100 colonies, but the numbers actually tested ranged between 68 and 101. Results were returned for a total of 1015 colonies. Laboratories were asked to select roughly half their colonies from membranes incubated at 37 °C, and half from those incubated at 44 °C. The overall returns were: 37 °C incubation; 542 colonies, 44 °C incubation; 472 colonies. In one case the information was not supplied. All isolates were subjected to all the confirmatory tests, irrespective of the original incubation temperature.

Lauryl 7:1 T:rose Mannitol Broth (LTMB) was used by all laboratories as a reference method, and all colonies were tested twice using this medium, with incubation at 37 °C and 44 °C on both occasions. The first occasion was immediately on picking the colony from the presumptive membrane test, and the second was after storage of the colony at 4 °C on a non-selective nutrient medium.

An initial examination of the data showed that a number of isolates gave a different pattern of results in LTMB after storage. There are numerous possible reasons for this, including the original colony not being a pure culture, and resuscitation of stressed organisms after laboratory subculture resulting in changes in biochemical characteristics. Whatever the reasons for the changes might be, any isolates giving inconsistent LTMB results on re-testing were considered invalid,
and were excluded from further analysis. The numbers of valid results are reported in later sections.

5.4 Treatment of results

In the following sections, the numbers of isolates giving positive and negative results in each test are recorded. To gain an idea of the relative performances of each test method, compared with the reference method, results for each test were tabulated as shown below:

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference method</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>eg LTMB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>a+c</td>
<td>b+d</td>
</tr>
</tbody>
</table>

From this table three values were calculated. The first (designated p1) was the probability of the test method giving a false negative result ("failure to alert"). This corresponds to b/(a+b), and is the proportion of the isolates which were positive by the reference method, which proved negative by the test method. The other two values (designated p2 and p3) reflected the incidence of false positive results, and this can be approached in two ways. Conceptually, the simpler of these is to calculate c/(a+c), that is the proportion of the isolates which were positive by the test method, which should have been negative according to the reference method. This gives the probability (p2) of a "false alarm" in practice, that is to say, if only the test method was carried out, in the absence of the reference method, it would give the probability of a positive result being incorrect. However, this value is highly dependent on the
conditions of the trial. This is particularly so in the present case, as all the colonies picked were acid-producing in the presumptive test, and so the probability of positive results in the confirmatory tests is high. This would tend to lower the relative incidence of "false alarms" calculated in this way, whereas if most isolates were giving negative results in the confirmatory tests, the incidence of such false alarms would become exaggeratedly high.

Therefore, as the colonies examined do not represent a random selection of positives and negatives, it is more realistic (though of less obvious practical use) to calculate \( c/(c+d) \), which is the proportion of the isolates which were negative by the reference method, which were incorrectly classified as positive by the test method. This value (p3) also represents a kind of "false alarm", but to distinguish it from the type already mentioned, it will be referred to as the incidence of "misidentified negatives". While it is theoretically a more satisfactory parameter, in practice it does not allow the assessment of the accuracy or otherwise of a positive result obtained using the test method only, without the reference method. Rather, in the event of obtaining a negative result using the reference method, it gives the probability that the test method would give a positive result for the same isolate.

This is not to say, of course, that the reference method is necessarily infallible, and if a method under test produces many more positive results than the reference method, the specificity and sensitivity of both need to be examined.
5.5

β-galactosidase (ONPG) test for coliform bacteria

Of the 1015 results collected, 953 isolates gave consistent results when re-tested in LTMB medium at 37 °C. Of these, 905 produced acid and gas, and were thus confirmed as coliform organisms by this test. Of the remainder, 47 were anaerogenic acid-producers, and one produced neither acid nor gas.

Table 2. Results of β-galactosidase tests

<table>
<thead>
<tr>
<th></th>
<th>β-gal and reference:</th>
<th>β-gal false:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>LTMB medium, 37 °C</td>
<td>905</td>
<td>48</td>
</tr>
<tr>
<td>RST medium, 2 h</td>
<td>280</td>
<td>673</td>
</tr>
<tr>
<td>RST medium, 6 h</td>
<td>748</td>
<td>205</td>
</tr>
<tr>
<td>RST medium, 24 h</td>
<td>944</td>
<td>9</td>
</tr>
<tr>
<td>RAPIDEC, 2 h</td>
<td>946</td>
<td>7</td>
</tr>
<tr>
<td>Lab-M, 2 h</td>
<td>755</td>
<td>198</td>
</tr>
<tr>
<td>Lab-M, 6 h</td>
<td>890</td>
<td>63</td>
</tr>
<tr>
<td>Oxoid, 2 h</td>
<td>881</td>
<td>72</td>
</tr>
<tr>
<td>Oxoid, 6 h</td>
<td>943</td>
<td>10</td>
</tr>
<tr>
<td>API RAPID 20E*</td>
<td>768</td>
<td>185</td>
</tr>
</tbody>
</table>

* Isolate identified as a coliform organism

The results for the various β-galactosidase tests are shown in Table 2. A positive result for LTMB indicates that the isolate showed both acid and gas production after 24 h incubation at 37 °C. The row labelled "API RAPID 20E" gives the number of isolates which were identified to at least the "acceptable" level as coliform organisms. Of the species encountered in this trial, this would mean that an isolate was identified as one of the following: *Citrobacter freundii*, *Escherichia coli*, *E. vulneris*, *Enterobacter aerogenes*, *Ent agglomerans*, *Ent cloacae*, *Ent taylorae*, *Enterobacter* species, *Klebsiella oxytoca* or *Kleb pneumoniae*. 
The Table shows that certain tests, notably RST medium after 2 and 6 hours, and the Lab-M tablets after 2 hours, gave high numbers of false negative results, indicating in each case that longer incubation was necessary. The apparently more sensitive of the β-galactosidase tests (RST after 24 h, RAPIDEC, and Oxoid discs after 6 h), gave more positive results overall than the reference method. To obtain a more objective view of the results, reference should be made to Table 3, which lists the parameters p1, p2 and p3, described earlier.

From the p1 column ("failure to alert"), it can be seen that RST medium after 2 hours would miss 72% of the isolates which were positive by the reference LTB test. After 6 hours incubation this had improved to 22%, but only after 24 hours had the failure to alert reduced to insignificant levels. Similarly the Lab-M tablets clearly needed longer than 2 hours to perform satisfactorily, though even after 6 hours they did not give as many positive results as the most sensitive methods. These, on the basis of the p1 calculation, were RST medium after 24 hours, RAPIDEC after 2 hours, and Oxoid discs after 6 hours. These only failed to alert in 1% of cases, and for ultimate rapidity, the RAPIDEC test was a clear winner.

This, however, is far from the whole story. The p2 column shows a consistent low level of "false alarms", but as explained earlier, these figures are artificially low because of the high overall proportion of positive results. This column is consequently best ignored for the β-galactosidase tests. The p3 column shows very high levels of "misidentified negatives" throughout. The only exception among the β-galactosidase tests was RST
Table 3. Relative performance of LTMB and \(\beta\)-galactosidase tests

Reference method: LTMB at 37 °C

<table>
<thead>
<tr>
<th>Method</th>
<th>p1</th>
<th>p2</th>
<th>p3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RST medium, 2 h</td>
<td>0.72</td>
<td>0.10</td>
<td>0.56</td>
</tr>
<tr>
<td>RST medium, 6 h</td>
<td>0.22</td>
<td>0.06</td>
<td>0.92</td>
</tr>
<tr>
<td>RST medium, 24 h</td>
<td>0.01</td>
<td>0.05</td>
<td>0.94</td>
</tr>
<tr>
<td>RAPIDEC, 2 h</td>
<td>0.01</td>
<td>0.05</td>
<td>0.96</td>
</tr>
<tr>
<td>Lab-M, 2 h</td>
<td>0.21</td>
<td>0.06</td>
<td>0.88</td>
</tr>
<tr>
<td>Lab-M, 6 h</td>
<td>0.07</td>
<td>0.05</td>
<td>0.98</td>
</tr>
<tr>
<td>Oxoid, 2 h</td>
<td>0.07</td>
<td>0.05</td>
<td>0.88</td>
</tr>
<tr>
<td>Oxoid, 6 h</td>
<td>0.01</td>
<td>0.05</td>
<td>0.98</td>
</tr>
<tr>
<td>API RAPID 20E*</td>
<td>0.18</td>
<td>0.03</td>
<td>0.52</td>
</tr>
</tbody>
</table>

p1 = "Failure to alert"
p2 = "False alarm"
p3 = "Hesitantly positives" (See text)
* Isolate identified as a coliform organism

after 2 hours, but this figure is misleading as relatively few positive results had been produced by this method after this incubation time. These high values in the p3 column mean that almost all the isolates which did not confirm as coliform organisms by the standard LTMB test, gave a positive \(\beta\)-galactosidase reaction.

This is not particularly surprising, as \(\beta\)-galactosidase is only one of the enzymes involved in lactose metabolism, and there is no reason why bacteria which cannot ferment lactose to produce acid, should not possess \(\beta\)-galactosidase activity. Only one of the 953 isolates under consideration here failed to produce acid in LTMB medium, while the maximum number of positives recorded by any of the \(\beta\)-galactosidase tests was 946 (RAPIDEC).

However, it is important to note that LTMB does not contain lactose, but mannitol instead, and this confuses the issue somewhat. Mannitol is claimed to give more copious gas production than lactose, but otherwise equivalent results in fermentation.
tests. However, it is unreasonable to expect exact equivalence between tests for fermentation of two different carbohydrates. Similarly it is perfectly possible for an organism to produce acid from mannitol, but not to possess \( \beta \)-galactosidase activity, as this enzyme is not involved in mannitol fermentation.

Overall, then, the situation is slightly confused. However, if an organism produces acid in a lactose fermentation test, or appears as an acid-producing colony in a presumptive membrane test, then it is virtually certain to possess \( \beta \)-galactosidase activity. The \( \beta \)-galactosidase test itself gives no information about gas production, but even if this is not considered important, the question must be asked as to whether it is worth performing a test where the outcome is almost certainly known beforehand.

5.6 API RAPID 20E for confirmation of coliform organisms

Table 3 shows that API RAPID 20E would fail to identify 18% of the isolates which were confirmed as coliforms by LTMB medium. The probability of "false alarms" was very low (but again this value is best ignored given the conditions of the trial). This method produced fewer "misidentified negatives" than the \( \beta \)-galactosidase tests, although these still amounted to over half of those which were negative by LTMB. These were mainly anaerogenic organisms, and while it is not intended to enter into a discussion of such things here, it is notable that gas production appears to be easily lost by some coliform organisms.

This aside, the data were re-examined to list all isolates which were coliform organisms according to the LTMB test, but were not identified by RAPID
20E. Overall 162 isolates fell into this category. Of these, 23 were identified as non-coliform organisms, and the rest were not identified at all. It is not unusual to encounter environmental strains which cannot be identified by methods such as API RAPID 20E, as the databases have been largely compiled from tests on clinical isolates. It is nevertheless worth cautioning users that such methods may miss a number of organisms which would be confirmed as coliform bacteria by the conventional tests.

5.7
\underbrace{\beta\text{-glucuronidase test}}_{\text{for } E\text{\_coli}}

A total of 799 isolates gave consistent results on re-testing in LTMB medium at 44 °C. Of these, 547 produced acid and gas, and also gave a positive indole reaction, thus confirming them as \textit{E\_coli} by this scheme. Of the rest, 7 were anaerogenic but indole positive, while 91 produced gas but were indole negative. Acid alone was produced by 47, and 107 gave negative reactions in all three tests.

The results for the various \(\beta\text{-glucuronidase tests}\) are shown in Table 4. A positive result for LTMB indicates that the isolate gave positive acid, gas and indole reactions after 24 h at 44 °C. A positive result using API RAPID 20E means that the isolate was identified to at least the "acceptable" level as \textit{E\_coli}.

While this table is not easily interpreted, it can nevertheless be seen that the Lab-M and Mast tests produced large numbers of false negative results when read after 2 hours, but better results after 6 hours. The RAPIDEC test, on the other hand, appeared not to need the extra incubation.
Table 4. Results of β-glucuronidase tests

<table>
<thead>
<tr>
<th></th>
<th>β-gluc and reference:</th>
<th>β-gluc false:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>LTMB medium, 44 °C</td>
<td>547</td>
<td>252</td>
</tr>
<tr>
<td>RAPIDEC, 2 h</td>
<td>530</td>
<td>269</td>
</tr>
<tr>
<td>RAPIDEC, 6 h</td>
<td>538</td>
<td>259</td>
</tr>
<tr>
<td>Lab-M, 2 h</td>
<td>253</td>
<td>546</td>
</tr>
<tr>
<td>Lab-M, 6</td>
<td>502</td>
<td>297</td>
</tr>
<tr>
<td>Mast, 2 h</td>
<td>209</td>
<td>575</td>
</tr>
<tr>
<td>Mast, 6 h</td>
<td>471</td>
<td>313</td>
</tr>
<tr>
<td>API RAPID 20E*</td>
<td>519</td>
<td>280</td>
</tr>
</tbody>
</table>

* Isolate identified as E coli

Table 5 shows the parameters p1, p2 and p3 calculated for the various β-glucuronidase tests. As expected from the examination of Table 4, the Lab-M and Mast tests gave a high probability of failure to alert after 2 hours incubation. This was improved after 6 hours, but neither was as good in this respect as the RAPIDEC test, or API RAPID 20E. The p2 column shows a fairly consistent low level of "false alarms". These values are slightly more meaningful than those calculated for the β-galactosidase tests, but should still be viewed with caution. It is only possible to say that, under the conditions of this trial, given an isolate showing positive β-glucuronidase activity, there was roughly a 10% probability that the organism would not confirm as E coli by the LTMB method.

More meaningful is the p3 column. Here the Lab-M and Mast 2-hour tests give low probabilities of misidentifying negatives, but this is misleading since these tests do not perform satisfactorily after this short incubation period. The remainder of the results are fairly consistent, and indicate
Table 5. Relative performance of LTMB and \(\beta\)-glucuronidase tests

Reference method: LTMB at 44 °C

<table>
<thead>
<tr>
<th>Method</th>
<th>p1</th>
<th>p2</th>
<th>p3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPIDEC, 2 h</td>
<td>0.12</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>RAPIDEC, 6 h</td>
<td>0.11</td>
<td>0.10</td>
<td>0.22</td>
</tr>
<tr>
<td>Lab-M, 2 h</td>
<td>0.58</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Lab-M, 6 h</td>
<td>0.18</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>Mast, 2 h</td>
<td>0.65</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>Mast, 6 h</td>
<td>0.22</td>
<td>0.11</td>
<td>0.21</td>
</tr>
<tr>
<td>API RAPID 20E*</td>
<td>0.12</td>
<td>0.16</td>
<td>0.18</td>
</tr>
</tbody>
</table>

p1 = "Failure to alert"
p2 = "False alarm"
p3 = "Misidentified negatives" (See text)
* Isolate identified as E. coli

that an isolate failing to confirm as E. coli by the LTMB test has roughly a 20% probability of giving a positive \(\beta\)-glucuronidase reaction.

The question remains as to whether the \(\beta\)-glucuronidase test can be used to replace the conventional confirmatory tests for E. coli. It is generally considered that the \(\beta\)-glucuronidase enzyme is specific, among the Enterobacteriaceae, to E. coli and some Shigellas. On the basis of the data obtained in this trial, with the LTMB test as the reference method, and using the RAPIDEC 6-hour results as giving the most sensitive enzyme test, 88% of the isolates confirming as E. coli possessed \(\beta\)-glucuronidase activity. Of the organisms which were shown not to be E. coli by LTMB, 22% also possessed the enzyme. These figures are less impressive than some which have been reported previously, but of course LTMB cannot be regarded as an absolute reference test for identifying E. coli. It would be tempting to use the API RAPID 20E results as the reference, but as shown in the next section, this method can sometimes fail to identify the presence of E. coli.
5.8
API RAPID 20E for confirmation of the presence of *E. coli*

Table 5 shows that there was a 12% probability of API RAPID 20E failing to identify isolates which were confirmed as *E. coli* by the LTMB method, a result similar to that obtained for the β-glucuronidase tests. The probability of "misidentified negatives" was also of the same order. An examination of the returns where isolates gave positive β-glucuronidase tests, but were not identified as *E. coli* by API RAPID 20E, showed that the latter method identified some isolates as coliform organisms other than *E. coli*, but in the majority (80%) of cases, no identification was made at all. Of this 80%, 67% were noted on the returns as having been satisfactorily identified on subsequent re-testing using API 20E, and all but one were identified as *E. coli*. The overall number in this category was not large, amounting to 5% of the 799 valid isolates, but it raises a suspicion that API RAPID 20E will miss certain strains of *E. coli* which are apparently easily identified by the full API 20E test.

It is somewhat disturbing to find that 20E and RAPID 20E can give different results for the same organism, but possibly this is a fact of numerical taxonomy which must be lived with. It is also worrying that revisions of the RAPID 20E database have resulted in some profiles giving different identifications depending on which profile index is used. Users of these identification kits should be alerted to the fact that it is necessary to keep up to date with all revisions. It has been seen in this trial that failure to do so leads to inconsistent and confusing results. Sadly it also means that strains of *E. coli* isolated in the past may no longer be the organisms they were thought to be.
6. CONCLUSIONS

1. The β-galactosidase test would appear to be a suitable means of determining rapidly whether an organism has the potential to produce acid from lactose. However, since it gives no information about gas production, the β-galactosidase test does little more than confirm that colonies identified as lactose fermenters on primary isolation media are indeed able to utilise this carbohydrate. The value of using this test instead of assuming that all lactose fermenting colonies on selective coliform media are in fact coliform organisms is therefore questionable.

2. If it is agreed that use of the β-galactosidase test has some merit, the RAPIDEC method would appear to be the most efficient in that it gives results in 2 hours with only a 1 per cent chance of failure to alert.

3. On the basis of the data obtained in this trial, the β-glucuronidase test would appear to be less specific for E. coli than has been previously reported. However, this may be due to the use of LTMB medium, rather than a more systematic scheme, as the reference method for identifying E. coli. In comparison to LTMB, the most sensitive β-glucuronidase test, RAPIDEC, failed to identify 11 per cent of the isolates as E. coli. Of the isolates not identified as E. coli by LTMB, 22 per cent possessed the β-glucuronidase enzyme.
4. On the basis of these results the LTMB and β-glucuronidase tests cannot be regarded as equivalent although the rapidity of the β-glucuronidase test may make it a useful additional test in certain operational situations.
REFERENCES


FENNELL H (1972) A single tube confirmatory test for E. coli at 44 °C. Water Treatment and Examination 21, 13-19.


APPENDIX A

Sample blank results sheet
RESULTS OF RAPID CONFIRMATION TRIAL

LABORATORY NAME  
LAB No.  
COLONY No.  
large  
small  
yellow  
pale  

SIZE AND COLOUR OF COLONY  

37°C membrane  
44°C membrane  

ORIGIN OF COLONY  
reservoir  
upland  
lowland  
river  
stream  
spring  
borehole  

TYPE OF WATER SOURCE (RAW)  
on-works  
consumer  
service  
res.  
other  

TYPE OF WATER SOURCE (FINAL)  

RESULTS IN USUAL CONFIRMATION MEDIUM:  
Medium  

24 hrs.  
growth @ 37°C gas @ 37°C Indole @ 4-  

6 hrs.  
growth @ 44°C gas @ 44°C Indole @ 4-  

24 hrs.  

RESULTS IN LTMB MEDIUM:  

24 hrs.  
growth @ 37°C gas @ 37°C Indole @ 4-  

6 hrs.  
growth @ 44°C gas @ 44°C Indole @ 4-  

6 hrs.  

24 hrs.  

* Remove 0.5ml aliquots to test for indole at 6hrs.

C. 04.10.72  
31.2.87  

A2
TESTS FROM NUTRIENT AGAR PLATES:

LABORATORY NAME:  
LAB No.:  
COLONY No.:  

RESULTS IN LIMB MEDIUM

<table>
<thead>
<tr>
<th>Growth @ 37°C gas @ 37°C Indole @ 37°C</th>
<th>6 hrs.</th>
<th>24 hrs.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Growth @ 44°C gas @ 44°C Indole @ 44°C</th>
<th>6 hrs.</th>
</tr>
</thead>
</table>

RESULTS IN RST MEDIUM

<table>
<thead>
<tr>
<th>β-gal 37°C indole 37°C β-gal 44°C indole 44°C</th>
<th>2 hrs.</th>
<th>6 hrs.</th>
<th>24 hrs.</th>
</tr>
</thead>
</table>

RESULTS IN API RAPIDEC 37°C

<table>
<thead>
<tr>
<th>β-gal</th>
<th>Indole</th>
<th>β-gluc</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hrs.</td>
<td>2 hrs.</td>
<td>6 hrs.</td>
</tr>
</tbody>
</table>

RESULTS FROM LAB MAL (Rosco) TABLETS

<table>
<thead>
<tr>
<th>β-gal</th>
<th>β-gluc</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hrs.</td>
<td>6 hrs.</td>
</tr>
</tbody>
</table>

RESULTS FROM MAST TABLETS

<table>
<thead>
<tr>
<th>β-gluc</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hrs.</td>
</tr>
<tr>
<td>6 hrs.</td>
</tr>
</tbody>
</table>

RESULTS FROM OXOID TABLETS

<table>
<thead>
<tr>
<th>β-gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hrs.</td>
</tr>
<tr>
<td>6 hrs.</td>
</tr>
<tr>
<td>Numerical Profile</td>
</tr>
<tr>
<td>Organism</td>
</tr>
</tbody>
</table>

RESULTS FROM Rapid 20E

<table>
<thead>
<tr>
<th>Numerical Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism</td>
</tr>
<tr>
<td>4 hrs.</td>
</tr>
</tbody>
</table>

RESULT OF API 20E

<table>
<thead>
<tr>
<th>Numerical Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism</td>
</tr>
<tr>
<td>24 hrs.</td>
</tr>
<tr>
<td>48 hrs.</td>
</tr>
</tbody>
</table>

Remove 0.5 ml aliquots to test for Indole at 2 hrs and 6 hrs.

C. BENTON  
23/10/87

A3
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