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Strathclyde Water Department

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

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CONFIRMING THE IDENTITY OF COLIFORM ORGANISMS

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SUMMARY

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.

CONTENTS

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:

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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 E coli after
they received their commercially produced LTMB. 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 LTMB, 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

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

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 o
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,   °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 1: 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:

```
              Test method:
                        |  +      -  |
Reference method    +   |  a      b  | a+b
       eg  LTMB          |           |
                    -   |  c      d  | c+d

                        | a+c    b+d | a+b+c+d
```

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 (p_3) 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

	+ve	−ve	β-gal and reference: +ve	β-gal and reference: −ve	β-gal false: +ve	β-gal false: −ve
LTMB medium, 37 °C	905	48				
RST medium, 2 h	280	673	253	21	27	652
RST medium, 6 h	748	205	704	4	44	201
RST medium, 24 h	944	9	899	3	45	6
RAPIDEC, 2 h	946	7	900	2	46	5
Lab-M, 2 h	755	198	713	6	42	192
Lab-M, 6 h	890	63	843	1	47	62
Oxoid, 2 h	881	72	839	6	42	66
Oxoid, 6 h	943	10	896	1	47	9
API RAPID 20E*	768	185	743	23	25	162

* 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 LTMB 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

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Table 3. Relative performance of LTMB and
           β-galactosidase tests

Reference method: LTMB at 37 °C

	p1	p2	p3
RST medium, 2 h	0.72	0.10	0.56
RST medium, 6 h	0.22	0.06	0.92
RST medium, 24 h	0.01	0.05	0.94
RAPIDEC, 2 h	0.01	0.05	0.96
Lab-M, 2 h	0.21	0.06	0.88
Lab-M, 6 h	0.07	0.05	0.98
Oxoid, 2 h	0.07	0.05	0.88
Oxoid, 6 h	0.01	0.05	0.98
API RAPID 20E*	0.18	0.03	0.52

p1 = "Failure to alert"
p2 = "False alarm"
p3 = "Misidentified negatives" (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 β-galactosidase reaction.

This is not particularly surprising, as
β-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 β-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 β-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 -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 β-galactosidase activity. The β-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 β-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
β-glucuronidase test for E 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 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 β-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 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

	+ve	-ve	β-gluc and reference: +ve	-ve	β-gluc false: +ve	-ve
LTMB medium, 44 °C	547	252				
RAPIDEC, 2 h	530	269	479	201	51	68
RAPIDEC, 6 h	538	259	484	197	54	62
Lab-M, 2 h	253	546	229	228	24	318
Lab-M, 6	502	297	451	201	51	96
Mast, 2 h	209	575	192	226	17	349
Mast, 6 h	471	313	420	192	51	121
API RAPID 20E*	519	280	480	213	39	67

* 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
β-glucuronidase tests

Reference method: LTMB at 44 °C

	p1	p2	p3
RAPIDEC, 2 h	0.12	0.10	0.20
RAPIDEC, 6 h	0.11	0.10	0.22
Lab-M, 2 h	0.58	0.10	0.10
Lab-M, 6 h	0.18	0.10	0.20
Mast, 2 h	0.65	0.08	0.07
Mast, 6 h	0.22	0.11	0.21
API RAPID 20E*	0.12	0.16	0.18

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 β-glucuronidase reaction.

The question remains as to whether the
β-glucuronidase test can be used to replace the
conventional confirmatory tests for E coli. It is
generally considered that the β-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
β-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.

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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.

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APPENDIX A

Sample blank results sheet

LABORATORY NAME [_____]   LAB No. [_____]   COLONY No. [_____]

SIZE AND COLOUR OF COLONY

large	small	yellow	pale

ORIGIN OF COLONY

37°membrane	44°membrane

TYPE OF WATER SOURCE (RAW)

reservoir
upland	lowland	river	stream	spring	boreho

TYPE OF WATER SOURCE (FINAL)

ex-works	consumer	service res.	other

RESULTS IN USUAL CONFIRMATION MEDIUM

MEDIUM [_____]

	* growth @ 37°C	gas @ 37°C	Indole @ 3
6hrs.			
24hrs.			

	growth @ 44°C	gas @ 44°C	Indole @ 4
6hrs.			
24hrs.			

RESULTS IN LTMB MEDIUM *

	growth @ 37°C	gas @ 37°C	Indole @ 3
6hrs.			
24hrs.			

	growth @ 44°C	gas @ 44°C	Indole @ 4
6hrs.			
24hrs.			

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

C.BENTON
23.12 87

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ABORATORY NAME [   ]   LAB No. [   ]   COLONY No. [·]

ESULTS IN LTMB MEDIUM   *

	growth @ 37°C	gas @ 37°C	Indole @ 3--
6hrs.			
24hrs.			

	growth @ 44°C	gas @ 44°C	Indole @ 44°
6hrs.			
24hrs.			

ESULTS IN RST MEDIUM   *

	β-gal 37°	indole 37°	β-gal 44°	indole 44°
2hrs.				
6hrs.				
24hrs.				

ESULTS IN API RAPIDEC 37°C

	β-gal	Indole
2hrs.		

	β-gluc
2hrs.	
6hrs.	

ESULTS from lab m (Rosco) TABLETS

	β-gal	β-gluc
2hrs.		
6hrs.		

ESULTS from MAST DISCS ~~TABLETS~~

	β-gluc
2hrs.	
6hrs.	

ESULTS from OXOID DISCS ~~TABLETS~~

	β-gal
2hrs.	
6hrs.	

ESULTS from RapiD 20E

	Numerical Profile
4hrs.	·

Organism

ESULT OF API 20E

	Numerical Profile
24hrs.	
48hrs.	

Organism

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

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