Letters in Applied Microbiology 1997, **24**, 286–290

An evaluation of the use of 4-methylumbelliferyl-β-D-glucuronide (MUG) in different solid media for the detection and enumeration of *Escherichia coli* in foods

P. Villari, M. Iannuzzo and I. Torre
Institute of Hygiene and Preventive Medicine, University 'Federico II' Medical School, Naples, Italy

1105/96: received and accepted 3 September 1996

P. VILLARI, M. IANNUZZO AND I. TORRE. 1997. The use of 4-methylumbelliferyl-β-D-glucuronide (MUG) in different solid media for the detection and enumeration of *Escherichia coli* in foods was evaluated by testing the effects of different substrate concentrations (50 or 100 μg ml^{-1}), incubation temperatures (37 or 41·5°C) and incubation times (8, 12, 24 and 48 h). Different kinds of foods, both naturally and artificially contaminated, were analysed. The use of selective media without differential substances and an incubation time of 24 h seem to be worthy of recommendation. In this case an incubation temperature of 37°C would be preferred and the MUG concentration could be reduced to 50 μg ml^{-1}. Incubation times shorter than 24 h, which may cause a loss of sensitivity, require higher incubation temperatures (41·5°C) and MUG concentration (100 μg ml^{-1}).

INTRODUCTION

The presence of *Escherichia coli* in foods is known to be the best indicator of faecal contamination. Mean counts of *E. coli* were recently proposed both as the base of a microbiological-quality grade of meat products and one of the microbiological tests in Hazard Analysis Critical Control Points procedures (Boyce *et al.* 1995; Guerrant and Theno 1995). In both cases, it is imperative that the micro-organism be readily quantifiable.

Fluorogenic detection and enumeration methods for *E. coli* in food and water have been developed. The assays are based mainly upon the activity of β-glucuronidase which cleaves the substrate 4-methylumbelliferyl-β-D-glucuronide. The parent substrate is colourless, but after the cleavage the free 4-methylumbelliferyl moiety fluoresces bluish when exposed to long-wave u.v. irradiation. In general, these methods have provided identification and enumeration systems that compare favourably with the conventional *E. coli* methods and shorten assay times to 24 h or less (Hofstra and Huisin't Veld 1988; Frampton and Restaino 1993; Blood and Curtis 1995). However, to our knowledge, no studies have been carried out on the efficacy of MUG in different culture media, testing the effects of different substrate concentrations, incubation temperatures and incubation times.

Correspondence to: Dr Paolo Villari, Istituto di Igiene e Medicina Preventiva, Universita' 'Federico II', Via S. Pansini 5, 80131 Naples, Italy.

This study was conducted to evaluate the use of MUG in different solid media and to optimize its use as a rapid method in food microbiology.

MATERIALS AND METHODS

Culture media

The culture media analysed included both (i) selective and differential agars and (ii) selective media without differential substances. Violet Red Bile Agar (VRBA) (Unipath), MacConkey Agar no. 3 (Unipath) and Tergitol 7 Agar (AT7) (Difco) were chosen as representative of the first group, whereas Tryptone Bile Agar (TBA) (Unipath), ECD Agar (Biolife) and MUG-7 Medium were selected as examples of the second group. Tryptone Soy Agar (TSA) (Unipath) was chosen as reference medium because of its lack of both selective and differential compounds.

Each medium was tested with two different concentrations of MUG (50 and 100 μg ml^{-1}) and at two incubation temperatures (37°C and 41·5°C). Readings were performed at 8, 12, 24 and 48 h of incubation.

MUG-7 medium was prepared as previously described (Sarhan and Foster 1991); all the other culture media were prepared as specified by manufacturers. MUG (Unipath) was added at the appropriate concentration after autoclaving and cooling the media to 50°C.

© 1997 The Society for Applied Bacteriology

Testing procedure

Twenty-five g of each food sample were stomached for 45 s in a Stomacher 400 (PBI) with 225 ml of phosphate-buffered saline (PBS, pH 7·2). Serial 10-fold dilutions in PBS were prepared and 1-ml aliquots of at least three dilutions were transferred to Petri dishes. Samples were plated out by the repair-enumeration procedure of Ray and Speck (1978), with MUG added at the same concentration to both layers of media. More precisely, a 1 ml portion of the appropriate dilution of the sample was mixed to 10 ml of melted TSA-MUG tempered to 50°C. Plates of TSA-MUG were incubated at room temperature for 1–2 h and then overlaid with 8 ml of the differential and/or selective medium also supplemented with MUG. The pour overlay method was preferred over the surface overlay method because more sample can be added per plate. Finally plates were incubated at 37° or 41·5°C up to 24–48 h. Plates following incubation were examined under long-wave u.v. irradiation (Fotodyne). Fluorescent and non-fluorescent colonies were identified using API systems (BioMérieux).

Study design

The effects of culture media, MUG concentrations, incubation temperatures and incubation times were evaluated against the following criteria: (i) sensitivity, recovery of fluorescent colonies from foods artificially contaminated with test suspensions of *E. coli*; (ii) selectivity, degree of reduction of background microbial flora of naturally contaminated foods; (iii) specificity, likelihood of false positives and false negatives on the ground of macroscopic examination of colonies recovered from naturally contaminated foods.

The sensitivity study was performed with 18 pre-sterilized foods (vegetables, meat and cheese products) artificially contaminated with 18 strains of *E. coli* isolated in this laboratory from different food samples by standard recovery methods not involving the use of MUG. Cultures were prepared by inoculating tubes containing 10 ml of Brain Heart Infusion Broth (Unipath) with strains of *E. coli* and incubated overnight at 37°C in order to reach an approximate concentration of 10^8–10^{10} cfu ml^{-1}. Test suspensions were prepared from these cultures by serial 10-fold dilutions in PBS. One ml portions of at least three consecutive dilutions were added to three aliquots of the 1:10 dilution of the food sample, in order to have at least 1 aliquot with a final concentration of 40–400 cfu ml^{-1}.

The selectivity study was carried out analysing the reduction of the background microbial flora in 21 naturally contaminated food samples (vegetables, meat and cheese products). More precisely, the degree of selectivity was calculated as the ratio between the cfu counts on the reference unselective medium (TSA) and the cfu counts observed on the differential and/or selective medium.

The specificity of the culture media was investigated in 21 naturally contaminated food samples (vegetables, meat and cheese products), by determining (i) the percentage of fluorescent colonies verified as *E. coli* and (ii) the percentage of non-fluorescent colonies which, in fact, were not *E. coli*.

Statistical analysis

One-way analysis of variance and the multiple comparison test of Bonferroni were used to compare means. Results were confirmed by parametric tests as Kruskal-Wallis. χ^2 test was used to compare proportions (Hamilton 1993).

In the sensitivity study a multiple linear regression analysis was performed to determine variables that were associated with higher cfu counts (Hamilton 1993). The explanatory variables were the following: (i) culture media; (ii) concentration of MUG (50 or 100 μg ml^{-1}); (iii) incubation temperature (37° or 41·5°C); (iv) incubation times (8, 12, 24 or 48 h).

Actual cfu counts instead of $\log_{10}$ counts were used since they were more normally distributed. All tests performed were two-sided. Data were analysed using the Stata software program (Stata Corp. 1993).

RESULTS

In the sensitivity study experiments were made with 18 strains of *E. coli*, used to experimentally contaminate 18 samples of different foods. For each experiment, the seven media analysed were incubated both at 37° and 41·5°C and tested with two concentrations of MUG (50 or 100 μg ml^{-1}). Readings were taken at 8, 12, 24 and 48 h of incubation. Therefore, 2016 cfu counts were globally available for the analysis.

Multiple linear regression is an efficient way to analyse simultaneously the effect of different explanatory variables on a dependent continuous variable (Hamilton 1993). Results of the model fitted to our data show that the observed cfu counts improve significantly with the increase of incubation time ($t = 36{\cdot}80$; $P < 0{\cdot}001$) and MUG concentration ($t = 2{\cdot}58$; $P = 0{\cdot}010$). The medium used in the test system exerted a strong and statistically significant influence ($t = -10{\cdot}34$; $P < 0{\cdot}001$), whereas no significant differences were found between incubation temperatures ($t = 0{\cdot}63$; $P = 0{\cdot}531$).

The significant differences among mean cfu counts observed with the seven media tested were confirmed by one-way analysis of variance ($F = 12{\cdot}94$, $P < 0{\cdot}0001$) and Kruskal-Wallis test ($\chi^2 = 79{\cdot}69$, $P = 0{\cdot}0001$). A Bonferroni multiple comparison test of differences of means showed that these differences arise mainly from the contrast between

media with and without differential compounds, with the higher cfu counts observed on selective (ECD agar, MUG-7 medium and TBA) and non-selective media (TSA).

Whereas the effect of media on cfu counts was observed for all incubation times, those of MUG concentration and incubation temperature varied with short (8 or 12 h) and long (24 or 48 h) incubation times. More precisely, the 41·5°C incubation temperature was able to improve cfu counts at 8 and 12 h, whereas for longer incubation times a slight but not significant improvement was observed at 37°C. At the same time, the beneficial effect of higher concentration of MUG was seen for short incubation times (8 or 12 h) and diminished as the incubation time increased (Table 1).

For four strains, experiments were repeated after heat stress (56°C × 10 min of stationary-phase cultures) and freeze stress (–10°C × 1 week). Findings obtained with uninjured *E. coli* (described above) on the effects of media, incubation temperatures, MUG concentrations and incubation times on *E. coli* cfu counts were confirmed, even if the results, given the smaller sample size, did not reach statistical significance (data not shown).

Twenty-one naturally contaminated foods were analysed in order to compare the selectivity and the specificity of the different media. In this case, given the results of the sensitivity study, only the MUG concentration of 100 μg ml^{-1} was used, whereas plates were incubated both at 37° and 41°C. Readings were performed after 24 h of incubation, since in the sensitivity study readings at 48 h were sometimes difficult because the fluorescent 4-methylumbelliferone released by GUD activity tended to diffuse throughout the medium.

The background contamination of the 21 food samples was between 10^4 and 10^6 cells g^{-1} when observed at 37°C and between 10^2 and 10^5 cells g^{-1} at 41·5°C. The selectivity of the different media was such that about a 10–100-fold reduction in the background flora was obtained on the different media relative to that observed on TSA (Table 2). The differences between the degree of selectivity of the different media were

Table 1 The effects of culture media, 4-methylumbelliferyl-β-D-glucuronide (MUG) concentrations, incubation temperatures and incubation times on the recovery of *Escherichia coli* strains from 18 artificially contaminated foods

		Mean *E. coli* cfu g^{-1} (fluorescent colonies)			
		MUG concentration			
		50 μg ml^{-1}		100 μg ml^{-1}	
		Incubation temperature		Incubation temperature	
Incubation times (h)	Media category*	37°C	41·5°C	37°C	41·5°C
8	1	10·0	589·4	5·6	282·2
	2	32·4	300·7	73·5	405·7
	3	2·2	34·8	49·1	50·0
	Total	16·3	228·8	53·3	235·6
12	1	1159·4	1423·3	1379·4	1620·0
	2	1273·3	1370·7	1399·8	1613·9
	3	783·0	668·7	1012·0	789·8
	Total	1046·9	1077·3	1230·7	1261·6
24	1	1877·8	1816·7	1717·8	1916·7
	2	1911·8	1779·8	1851·8	1891·7
	3	1498·0	1288·7	1553·9	1382·2
	Total	1729·6	1574·6	1705·0	1676·9
48	1	1779·4	2003·9	1919·4	2027·7
	2	1801·7	1877·0	1936·7	1971·7
	3	1700·4	1410·5	1728·1	1548·1
	Total	1755·1	1695·2	1844·8	1798·1

*1, Medium without different and selective substances (TSA); 2, selective media (ECD agar, MUG-7 medium, TBA); 3, selective and differential media (VRBA, MacConkey agar no. 3, AT7 agar).

Table 2 Selectivity of 4-methylumbelliferyl-β-D-glucuronide (MUG) solid media

Media*	Reduction of background microbial flora† (means of 21 naturally contaminated foods)	
	37°C	41·5°C
MUG-7 medium	44·69	105·35
TBA	55·15	15·38
ECD agar	44·86	54·33
VRBA	35·53	56·80
MacConkey agar no. 3	65·51	41·11
AT7 agar	48·08	18·22

*All media contained a MUG concentration of 100 μg ml^{-1} and all readings were performed at 24 h.
†Expressed as the ratio between cfu count g^{-1} observed on TSA and cfu count g^{-1} observed on differential and/or selective medium.

not significant at both temperatures when analysed with parametric and non-parametric tests.

Fluorescent colonies (248) and non-fluorescent (212) colonies isolated from the 21 food samples on the different media were identified at the species level in order to test the likelihood of false positives and false negatives at the macroscopic examination of colonies. 96·8% of fluorescent colonies were verified as *E. coli* and only 6·9% of non-fluorescent colonies were identified as *E. coli* (Table 3). Selective and differential media (VRBA, MacConkey agar no. 3 and AT7) seem to have the tendency (not significant) to give more false negatives (non-fluorescent colonies which were actually *E. coli*), in agreement with the results of the sensitivity study. Moreover, higher cfu counts of fluorescent colonies were observed on unselective (TSA) and selective media (ECD agar, MUG-7 medium and TBA) (data not shown).

DISCUSSION

We evaluated the incorporation of MUG in different solid media which had already been tested, in some cases with modifications, for the identification or the enumeration of *E. coli* from different sources (Feng and Hartmann 1982; Alvarez 1984; Trepeta and Edberg 1984; Damaré *et al.* 1984; Heizmann *et al.* 1988; Weiss and Humber 1988; Ogden and Watt 1991; Schets and Havelaar 1991; Sarhan and Foster 1991). These studies showed in most cases that the incorporation of MUG into solid coliform media is an attractive alternative to current detection methods. Our study was performed to optimize the use of MUG as a rapid method in microbiological quality control in the food industry by testing the effects of different culture media, substrate concentrations, incubation temperatures and incubation times. The results show first of all that media without differential substances gave higher counts of *E. coli* showing the typical fluorescence. The acidification of the agar surrounding *E. coli* colonies in lactose-based media negatively affects the discrimination of MUG hydrolysing colonies. This is consistent with the finding which documented a more pronounced fluorescence from MUG hydrolysis at alkaline pH (Frampton and Restaino 1993).

Table 3 Verification of fluorescent and non-fluorescent colonies on 4-methylumbelliferyl-β-D-glucuronide (MUG) solid media

Media*		No. of fluorescent colonies		No. of non-fluorescent colonies	
		Examined	Verified (%)†	Examined	Verified (%)†
MUG-7 medium	37°C	21	20 (95·24)	18	1 (5·55)
	41·5°C	20	20 (100)	18	0 (0·00)
TBA	37°C	23	21 (91·30)	19	0 (0·00)
	41·5°C	19	18 (94·77)	18	0 (0·00)
ECD agar	37°C	22	22 (100)	19	0 (0·00)
	41·5°C	20	19 (95·00)	18	1 (5·55)
VRBA	37°C	21	20 (95·24)	16	2 (12·50)
	41·5°C	20	19 (95·00)	17	1 (5·88)
MacConkey agar no. 3	37°C	22	20 (90·90)	18	2 (11·11)
	41·5°C	19	18 (94·74)	16	2 (12·50)
AT7 agar	37°C	22	18 (81·82)	19	2 (10·52)
	41·5°C	19	17 (89·47)	16	3 (18·75)

*All media contained a MUG concentration of 100 μg ml^{-1} and all readings were performed at 24 h.
†Verified as *Escherichia coli*.

Readings at 24 h seem to be worthy of recommendation. In many cases the prolongation of incubation to 48 h produced a diffuse fluorescence of the media that may render counts of β-glucuronidase-positive colonies in mixed cultures difficult to read. On the other hand, the use of incubation times shorter than 24 h may cause a loss of sensitivity. In our experience with selective media without differential substances, readings at 8 and 12 h gave recoveries of fluorescent colonies — expressed as percentages of cfu counts at 24 h — of 10–15% (8 h) and 80–85% (12 h). If this is an acceptable figure in some settings, the MUG concentration of 100 μg ml^{-1} and an incubation temperature of 41·5°C would be advisable. In the case of readings at 24 h an incubation temperature of 37°C would be preferred and the MUG concentration could be reduced to 50 μg ml^{-1}, especially when media without differential substances are used.

The performance and the value of MUG as an indicator cannot be separated from the selective aspects of the medium since glucuronidase activity is not unique to *E. coli*. The results presented here show that all media tested are able to reduce the background contamination flora of naturally contaminated foods by at least one order of magnitude (10-fold). Moreover, only few fluorescent colonies were not *E. coli*, and less than 7% of non-fluorescent colonies were actually *E. coli*, in agreement with most studies previously performed (Frampton and Restaino 1993).

In conclusion, the appropriate selection of media compounds, concentration of substrate, incubation temperature and incubation time is essential for creating the efficacy of MUG in detection and enumeration of *E. coli* from foods. Whereas the use of selective media without differential substances is strongly recommended, the choice of the incubation time, incubation temperature and concentration of substrate may depend on the purpose of analysis and its required level of sensitivity.

REFERENCES

Alvarez, R.J. (1984) Use of fluorogenic assays for the enumeration of *Escherichia coli* from selected seafoods. *Journal of Food Science* **49**, 1186–1187.

Blood, R.M. and Curtis, G.D.W. (1995) Media for total Enterobacteriaceae, coliform and *Escherichia coli*. *International Journal of Food Microbiology* **26**, 93–115.

Boyce, T.G., Swerdlow, D.L. and Griffin, P.M. (1995) *E. coli* O157:H7, *Lancet* **333**, 1712.

Damaré, J.M., Campbell, D.F. and Johnston, R.W. (1985) Simplified direct plating method for enhanced recovery of *Escherichia coli* in food. *Journal of Food Science* **50**, 1736–1746.

Feng, P.C.S. and Hartman, P.A. (1982) Fluorogenic assay for immediate confirmation of *Escherichia coli*. *Applied and Environmental Microbiology* **43**, 1320–1329.

Frampton, E.W. and Restaino, L. (1993) Methods of *Escherichia coli* in food, water and clinical samples based on β-glucuronidase detection. *Journal of Applied Bacteriology* **74**, 223–233.

Guerrant, R.L. and Theno, D.M. (1995) *E. coli* O157:H7. *Lancet* **333**, 1711–1712.

Hamilton, L.C. (1993) *Statistics with Stata 3*. Belmont, CA: Duxbury Press.

Heizmann, W., Doller, P.C., Gutbrod, B. and Werner, H. (1988) Rapid identification of *Escherichia coli* by fluorocult media and positive indole reaction. *Journal of Clinical Microbiology* **26**, 2682–2684.

Hofstra, H. and Huis in't Veld, J.H.J. (1988) Methods for detection and isolation of *Escherichia coli* including pathogenic strains. *Journal of Applied Bacteriology Symposium Supplement* 197S–212S.

Ogden, I.D. and Watt, A.J. (1991) An evaluation of fluorogenic and chromogenic assays for the direct enumeration of *Escherichia coli*. *Letters in Applied Microbiology* **13**, 212–215.

Ray, B. and Speck, M.L. (1978) Plating procedure for the enumeration of coliform from dairy products. *Applied and Environmental Microbiology* **35**, 820–822.

Sarhan, H.R. and Foster, H.A. (1991) A rapid fluorogenic method for the detection of *Escherichia coli* by the production of β-glucuronidase. *Journal of Applied Bacteriology* **70**, 394–400.

Schets, F.M. and Havelaar, A.H. (1991) Comparison of indole production and β-glucuronidase activity for the detection of *Escherichia coli* in membrane filtration methods. *Letters in Applied Microbiology* **13**, 272–274.

Stata Corp. (1993) *Stata Reference Manual: release 3.1 (6th edn)*. College Station, TX.

Trepeta, R.W. and Edberg, S.C. (1984) Methylumbelliferyl-β-D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli*. *Journal of Clinical Microbiology* **19**, 172–174.

Weiss, L.H. and Humber, J. (1988) Evaluation of a 24-hour fluorogenic assay for the enumeration of *Escherichia coli* from foods. *Journal of Food Protection* **51**, 766–769.