False-Positive Fluorescence by Pink Salmon Tissue and Staphylococci in a Rapid Test for Escherichia coli†

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MS 97-300: Received 15 December 1997/Accepted 20 February 1998

ABSTRACT

Fluorescence from 4-methylumbelliferyl-β-D-glucuronide (MUG) hydrolysis is a common, rapid method for determining Escherichia coli in water and food. False-positive fluorescence occurred when either pink salmon fillets were tested or β-glucuronidase-positive Staphylococcus species were present in other fish products. Salmon fillet, E. coli, S. xylosus, and S. warneri produced 2, 17, 39, and 43 nmol of 4-methylumbelliferone per ml, respectively, in a one-step detection broth (lauryl salts tryptose broth with MUG) for E. coli after 48 h at 35°C. These false-positive reactions need to be considered when testing fish products, especially those contaminated through human handling.

Hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG) is used as a rapid, visual indicator for detecting Escherichia coli (6). Commercially available media and kits using MUG have been developed for testing food and water. False-negative fluorescence is a common trait among enteropathogenic E. coli strains (2). Conversely, false-positive fluorescence is caused by less than 5% of bacterial species isolated from food and results from the presence of Shigella, Salmonella, Klebsiella, Streptococcus, and Staphylococcus (3, 5, 9, 11, 12, 15). False-positive reactions also occur from β-glucuronidase activity by nonmicrobial sources, such as marine plants, shellfish, and particularly oysters (4, 8, 10, 14).

In our laboratory, preliminary tests of pink salmon tissue indicated 4-methylumbelliferone (4-MU) fluorescence occurred in the absence of β-glucuronidase-producing bacteria. Additionally, β-glucuronidase-positive Staphylococcus species were isolated from pink salmon fillets and fish meal (unpublished data) and smoked salmon strips (7) during routine analysis for E. coli. Both results can complicate seafood microbiological analysis and can lead to unnecessary destruction of product due to false implication of E. coli. We investigated the false-positive (visual) reactions, occurring after incubation of salmon preparations for microbiological analysis, by confirming the reactions spectrofluorometrically. The objective was to quantify 4-MU due to putative β-glucuronidase activity in pink salmon tissue and in two Staphylococcus species by the one-step MUG test used routinely for E. coli.

MATERIALS AND METHODS

Sample preparation and microbial analysis. Several pink salmon (Oncorhynchus gorbuscha) fillets were obtained from local seafood processing plants. Fillets were cut into 25-g portions, diluted 10-fold in sterile Butterfield's buffer, stomached (Model 400, Tekmar Co., Cincinnati, Ohio) intermittently for 2 min at 25°C, and serially diluted. Triplicate tubes containing 10 ml of lauryl salts tryptose broth (LST; Difco Laboratories, Detroit, Mich.) supplemented with 100 μg of MUG (Hach Co., Loveland, Colo.) per ml and triplicate tubes containing 10 ml of EC medium (Difco) containing MUG were inoculated with 1.0-ml dilutions. Tubes containing LST + MUG were incubated at 35°C for 48 h, and EC + MUG tubes were incubated at 44.5°C for 24 h. Cultures were checked for β-glucuronidase activity by observing 4-MU fluorescence in tubes exposed to a longwave lamp (366 nm; Model UVL-56, UVP Inc., San Gabriel, Calif.). Glucuronidase-positive E. coli and Staphylococcus species were confirmed after observing fluorescent halos surrounding colonies under longwave UV light on MUG-supplemented plate count agar (PCA + MUG; Difco) and Baird-Parker agar (BP + MUG; Difco). Staphylococcal species were identified using STAPH-IDENT and STAPH-Trac (API Analytab Products, Hazelwood, Mo.).

Effects of sample preparation and incubation conditions on 4-MU fluorescence production. A series of experiments was conducted to demonstrate the effects on production of 4-MU fluorescence. Sample size varied from 10 to 50 g prior to stomaching in sterile Butterfield’s buffer. A blender (Model 890-23J Osterizer Mini-Blend, Oster Co., Milwaukee, Wis.) was used to grind tissue intermittently for 2 min at 25°C to compare with stomaching. Fillet homogenates were membrane filtered (0.2 μm; Gelman Sciences Inc., Ann Arbor, Mich.) and were added to LST + MUG and EC + MUG broths. Heat lability of the putative enzyme in salmon fillet was confirmed by autoclaving (121°C, 15 min) prior to sampling. Incubation temperature of fillet homogenate in LST + MUG was varied from 25 to 60°C for 48 h, and incubation time was varied from 24 to 72 h at 35°C. Fillet concentrations in the initial homogenate in buffer were varied from 5 to 50% prior to stomaching.

4-MU fluorescence assay. Optimum excitation and emission wavelengths (λ) were conducted on replicate assays of 0.5 μM 4-MU (catalog no. M-1508, Sigma Chemical Co., St. Louis, Mo.) prepared fresh in distilled water. One-milliliter aliquots of culture broths were microcentrifuged (13,000 × g, 4 min, 25°C; Model
Micro MB, International Equipment Co., Needham Heights, Mass.), and paired supernatants were combined. Two milliliters, diluted in sterile LST + MUG if necessary, was injected through a 10-μl flow cell in a dual monochromator fluorescence detector (Model FD-300, SpectroVision, Chelmsford, Mass.). The fluorescence value was subtracted from the value for supernatant from uninoculated broth or appropriate broth dilution (control) and was corrected for dilution factor. Fluorescent end product in the supernatants and dilutions was quantified against a standard curve of 0.5 mM 4-MU prepared fresh in sterile LST + MUG.

**Bacteria-free salmon tissue.** The skin of headed and gutted salmon was doused in ethanol and flamed briefly. Fillets were aseptically excised and subdivided into 10-g portions. Sterile tissues were tested for heterotrophic bacteria by aerobic plate counts. Serially diluted samples were streak plated on PCA and were incubated at 25°C for 72 h. Absence of coliforms or staphylococci was checked using LST (Difco) containing 10% NaCl, respectively, that was incubated at 35°C for 48 h.

**Inocula preparation and sampling.** Overnight cultures (35°C) of individual strains of coliform and staphylococcal species, previously isolated from seafood products, except for *E. coli* O157:H7 (ATCC 43895; American Type Culture Collection, Rockville, Md.), were prepared in 3 ml of brain heart infusion (Difco). Fillet portions were inoculated with 0.2 ml of culture, and the samples were diluted and inoculated in triplicate LST + MUG tubes. Spent media were microcentrifuged and assayed for 4-MU.

**Statistical analysis.** One-way analysis of variance (ANOVA), least significant difference (LSD), and coefficient of determination ($r^2$) for linear regression were calculated for individual experiments (1, 13).

**RESULTS AND DISCUSSION**

A rapid, one-step fluorescent detection method is beneficial in reducing laboratory time to within 1 day for determining the presence of *E. coli*. The use of LST + MUG for solely confirming *E. coli* in foods was originally proposed by Feng and Hartman (5) and was evaluated by Robison (11). However, in assessing the microbial quality of seafood processed in Alaska, we observed that pink salmon fillets produced UV fluorescence when incubated in media containing MUG. Some samples contained coliform bacteria, but *E. coli* was not isolated. False-positive fluorescence occurred in the lowest dilution ($10^{-1}$) tubes used in most probable number tests for presumptive coliforms (LST + MUG) and fecal coliforms (EC + MUG). Autofluorescence and false-positive fluorescence occurred when finfish and shellfish samples were tested using MUG-based media for *E. coli* (8, 10, 14).

Fluorescence intensities were observed and corresponded to levels of 4-MU hydrolyzed from MUG. Since the visual results of MUG hydrolysis are qualitative, 4-MU produced from pink salmon tissue in MUG-containing media was quantified. A standard curve of 4-MU based on fluorometric readings (maxima of 386 nm excitation $\lambda$ and 450 nm emission $\lambda$; Fig. 1) was linear to 1 μM (Fig. 2).

Fluorescence under UV was apparent ("+" by observation) when 2 to 10 nmol of 4-MU per ml was produced by pink salmon tissue (10% wt/vol) in broth. Initial filament samples of 10, 25, and 50 g in the 10% homogenates added to LST + MUG resulted in 3.3 ± 1.6, 4.2 ± 0.8, and 2.8 ± 0.3 nmol of 4-MU per ml, respectively, although the

**FIGURE 1.** (a) Fluorescence of 4-MU at various excitation wavelengths ($\lambda$). Emission $\lambda$ set at 445 nm. (b) Fluorescence of 4-MU at various emission wavelengths ($\lambda$). Excitation $\lambda$ set at 386 nm. Error bars are for triplicate assays for excitation $\lambda$ (383 to 388 nm) in (a) and triplicate or quadruplicate assays for emission $\lambda$ (446 to 452 nm) and duplicate assays at the other $\lambda$ in (b).
FALSE-POSITIVE FLUORESCENCE FROM MUG

Differences were not significant ($P > 0.05$, ANOVA). The lower amount of fluorescent end product, when 50 g of fillet was tested, may have resulted from incomplete stomaching of the 500-ml homogenate.

Less than twice as much 4-MU was formed by the salmon tissue (3.9 ± 0.6 versus 6.5 ± 0.5 nmol of 4-MU per ml) when stomached, rather than blended, but the differences were significant ($P < 0.05$, ANOVA). Shearing of salmon tissue by blender blades may have been more efficient in releasing the salmon enzyme than stomaching, which is used commonly to provide homogeneous mixtures for microbiological analysis.

Four times less fluorescence in LST + MUG and EC + MUG occurred when membrane-filtered fillet homogenate was compared to unfiltered homogenate (data not shown; $P < 0.01$, LSD). The putative salmon enzyme was suggested to be cell bound or contained in cells. No fluorescence occurred when tissue was autoclaved due to inactivation of the enzyme.

Incubation temperature, rather than medium formulation, was an essential parameter for β-glucuronidase activity. Fillet homogenate (10%) added to LST + MUG resulted in four times more 4-MU when incubated at 44.5°C than at 35°C for 24 h (data not shown; $P < 0.001$, ANOVA). No detectable fluorescence was produced in LST + MUG containing fillet homogenate incubated at 25 or 60°C for 48 h. Release of 4-MU increased significantly ($P < 0.005$, LSD) from 1.3 ± 0.4 to 3.6 ± 0.3 to 6.6 ± 0.8 nmol/ml when fillet homogenates were incubated in LST + MUG at 35°C for 24, 48, and 72 h, respectively, and all were “+” fluorescent.

Fluorescence increased linearly ($r^2 = 0.986$) when the fillet concentration was increased in the initial homogenate (Fig. 3). Subjective observations of fluorescence were “−” for 0%, “±” for 5%, “+” for 10 to 20%, and “++” for 33 to 50% fillet concentrations.

Noncoliform bacteria in pink salmon fillets, fish meal, and smoked salmon strips were isolated from LST + MUG and were identified as β-glucuronidase-positive S. warneri and S. xylosus. Staphylococci are common bacteria in the microbial flora of human skin and can be transferred through human contact during food processing. Colonies and surrounding zones were fluorescent under longwave UV light on BP + MUG. Strong fluorescence was observed for two strains of E. coli, S. warneri, and S. xylosus in LST + MUG (Table 1) and by colonies on PCA + MUG. However, EC + MUG incubated at 44.5°C inhibited both Staphylococcus species, and the tubes showed no UV fluorescence.

When sterile pink salmon fillet homogenates (aerobic plate counts <10²/g; coliforms, E. coli, and S. aureus <3/g) were inoculated with other coliform bacteria in LST + MUG, all samples produced 2 to 10 nmol of 4-MU per ml and had visually equivalent fluorescence. Significant differences ($P < 0.005$, LSD) occurred between mean fluorescent values for the two staphylococcal and E. coli strains, Citrobacter freundii, and the uninoculated fillets (Table 1). Besides the two staphylococcal species used in the present study, S. haemolyticus, S. simulans, and S. cohnii produce false-positive reactions in LST + MUG (9).

In summary, false-positive fluorescent reactions need to be considered when testing fish and products that may contain endogenous β-glucuronidase-like enzymes or β-gluc-
FIGURE 3. Fluorescent end product in supernatants from different initial percentages of fillet homogenates in LST + MUG broth (35°C, 48 h). Error bars are for triplicate assays, except for the control and a single assay performed for the 10% fillet homogenate.

![Graph showing fluorescent end product in supernatants from different initial percentages of fillet homogenates in LST + MUG broth.](image)

TABLE 1. Fluorescence values for supernatants from pink salmon fillet and bacterial cultures incubated in LST + MUG broth

<table>
<thead>
<tr>
<th>Culture</th>
<th>Fluorescence value (nmol of 4-MU per ml)</th>
<th>Fluorescencea</th>
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</thead>
<tbody>
<tr>
<td><em>Staphylococcus warneri</em> 11E3</td>
<td>43.3 ± 0.7</td>
<td>++</td>
</tr>
<tr>
<td><em>Staphylococcus xylosus</em> 13A4</td>
<td>39.4 ± 1.1</td>
<td>++</td>
</tr>
<tr>
<td><em>Escherichia coli</em> 1D3</td>
<td>18.6 ± 0.6</td>
<td>++</td>
</tr>
<tr>
<td><em>Escherichia coli</em> 11B5</td>
<td>14.8 ± 2.6</td>
<td>++</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> 13B3</td>
<td>8.0 ± 0.4</td>
<td>+</td>
</tr>
<tr>
<td><em>Citrobacter species</em> (CDC-17)</td>
<td>4.2 ± 0.3</td>
<td>+</td>
</tr>
<tr>
<td>Other bacteriab</td>
<td>0.5–3.5 ± 0.2–1.5</td>
<td>+</td>
</tr>
<tr>
<td>None (fish only)</td>
<td>1.8 ± 0.5</td>
<td>+</td>
</tr>
<tr>
<td>None (broth only)</td>
<td>&lt;0.05</td>
<td>-</td>
</tr>
</tbody>
</table>

a Strong (++), bright (+), and none (−).

In this study, we showed 4-MU production was influenced by sample preparation and incubation conditions used in standard microbiological analysis. The compound was quantified spectrofluorometrically and was correlated with subjective observations of fluorescent broth containing pink salmon tissue and/or β-glucuronidase-positive bacteria. Two potential situations can lead the laboratory analyst to erroneously conclude that *Escherichia coli* is present in the one-step, visual LST + MUG test. First, contamination of pink salmon fillets by β-glucuronidase-negative coliforms and fecal coliforms may result in gas-positive, UV fluorescence. Second, foods contaminated through human handling with a combination of β-glucuronidase–positive staphylococci and β-glucuronidase–negative coliforms may give the same visual reaction. One solution is to subculture fluorescent tubes into EC + MUG (44°C, 24 h) to confirm glucuronidase-positive *E. coli* (8).

REFERENCES

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