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Environmental Monitoring

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ABSTRACT

MUG negative *Escherichia coli* are a small fraction (2.5%) of the total *E. coli* in Kentucky groundwater samples. It is unlikely that they alone will cause a significant potential to underestimate fecal contamination using MUG as the primary criterion for that assessment. An unresolved question is how effectively MUG-based, defined-substrate tests address false negative water samples containing MUG positive *E. coli*.

CONSIDERABLE DEBATE has surrounded the appropriate diagnostic tests to rapidly and accurately determine fecal contamination of drinking water supplies. The EPA has promulgated regulations which make *Escherichia coli* enumeration the principle criterion on which to assess water quality (9). β-glucuronidase activity, as indicated by the metabolism of 4-methylumbelliferyl β-D-glucuronide (MUG), has been recommended as a sensitive metabolic indicator of *E. coli* (8,10).

Chang et al. (4) indicated that up to 30% of *E. coli* isolates from human fecal samples were MUG negative. This would cause an unacceptable risk of underestimating *E. coli* populations. Their work has been contradicted by several other groups who contend that the true incidence of MUG negative *E. coli* is much smaller (14,15). Clark et al. (6) using two commercial MUG-based tests, found a higher incidence of false negative samples in treated water compared to untreated water. Most, but not all of the *E. coli* isolated from these false negative samples expressed a MUG positive phenotype during subsequent incubation. Covert et al. (7) also observed recovery of MUG positive phenotypes in *E. coli* isolated from false negative water samples. This implies that sublethal exposure of *E. coli* to water treatment affects the permeability of *E. coli* to MUG or the expression of glucuronidase activity.

Little work has been done on the frequency of MUG negative *E. coli* in untreated (i.e., unchlorinated) groundwater supplies, which make up the bulk of drinking water for many regions, including Kentucky. Shadix and Rice (16) observed a frequency of 0 to 25% MUG negative *E. coli* in environmental water samples from various sources with known fecal contamination.

Our principle objective was to determine the fre-

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Abbreviations: 4-methylumbelliferyl β-D-glucuronide; EPA, Environmental Protection Agency; ONPG, O-Nitrophenyl β-D-galatopyranoside; IPTG, isopropyl thiogalactoside; IMViC, Indole, Methyl Red, Voges-Proskauer, Citrate.

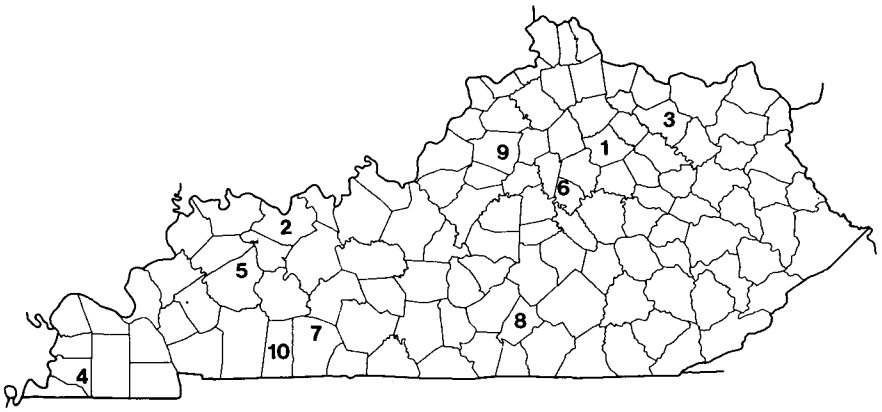

Fig. 1. Geographic distribution of groundwater sample sites: 1. Bourbon County; 2. Daviess County; 3. Fleming County; 4. Hickman County; 5. Hopkins County; 6. Jessamine County; 7. Logan County; 8. Russell County; 9. Shelby County; 10. Todd Country.

quency of MUG negative *E. coli* in groundwater samples typical of those found in Kentucky, and identify if there was the potential to seriously underestimate fecal contamination using MUG response as the sole criterion of water quality assessment. Our study is unique in that we examined groundwater samples (principally springs) at agricultural sites over a broad geographic area without regard to prior evidence of fecal contamination. We also tested the utility of a developmental defined-substrate (ONPG (O-Nitrophenyl β-D-galatopyranoside)-MUG) medium, Colitag (5), in the culture, and detection of *E. coli*.

MATERIALS AND METHODS

Over 100 groundwater samples were collected in 10 Kentucky counties from December 1991 to March 1992 as part of a larger assessment of nonpoint-source agricultural pollution of groundwater being done at the University of Kentucky (17). The distribution of the sampling sites, which included springs, streams, and former drinking water wells, is shown in Fig. 1. These sites are all representative of groundwater sources with the potential, if not the evidence, for point and nonpoint-source agricultural pollution from domestic and pastoral fecal contamination via failed septic systems, surface runoff, and direct deposition.

Escherichia coli were enumerated in these groundwater samples using standard membrane filtration techniques and incubation at 44.5 °C on MFC agar (DIFCO Laboratories, Detroit, MI). Once enumerated, individual isolated colonies putatively identified as *E. coli*, based on colony morphology and color on MFC agar (DIFCO), were randomly selected and inoculated into individual culturette tubes containing 4 mL Colitag medium. The composition of Colitag medium (5) was (g L^{-1}): ONPG (0.1); sodium lauryl sulfate (0.1); IPTG (Isopropyl thiogalactoside) (0.1); tryptophane (1.0); tryptose (2.5); MUG (0.05); $(NH_4)_2SO_4$ (2.5); NaCl (2.9); Na_2HPO_4 (3.0); KH_2PO_4 (0.5); $MgSO_4$ (0.1).

After incubating the tubes 22.5 h at 44.5°C, ONPG hydrolysis was noted by formation of the yellow colored metabolite *o*-nitrophenol. MUG hydrolysis was detected by fluorescence in culturette tubes exposed to a hand-held long wavelength ultraviolet light compared with MUG positive (*E. coli* ATCC 25922) and MUG negative (*Klebsiella pneumoniae* ATCC 13883) controls. Indole production was observed by dispensing 100 to 200 μL Kovac's reagent into each tube and observing the formation of a red ring.

The ONPG negative tubes were rejected outright as non *E. coli* isolates. Occasionally, fluorescent ONPG negative tubes were observed. These were attributed to growth of heat tolerant fluorescent pseudomonads and could be readily distinguished from MUG positive *E. coli* cultures based on the intensity of fluorescence (milky white vs. intense blue) and by plating on MacConkey's agar (American Public Health Association, 1992). An IMViC (Indole, Methyl Red, Voges-Proskauer, Citrate) test was performed on all ONPG positive isolates to further confirm them as *E. coli*. Isolates that had an IMViC response of + + − − were assumed to be putative *E. coli*. All putatively MUG negative and indole negative *E. coli* were recultured in Colitag to verify the initial MUG negative phenotype and subsequently identified by defined-substrate use patterns using commercially prepared microtiter plates (Biolog Inc, Hayward, CA).

To test effects of cell permeability on MUG response, representative MUG negative and MUG positive *E. coli* were grown overnight in 20 mL EC broth (DIFCO) without MUG at 35 °C. The cultures were harvested by centrifugation and resuspended in 4 mL physiological saline. Each culture was divided into two lots. One lot was sonicated with a 25 W sonicator using a 2 mm probe at 60% full power for 5 min in bursts of 1 min followed by cooling on ice for 30 s. The other lot was not sonicated. From both lots, 0.5 mL of resuspended cells was dispensed into three nonfluorescing culture tubes, amended with 25 μg MUG, and incubated at 44 °C. Periodically, a longwave ultraviolet light was used to look for fluorescence in the tubes.

RESULTS

Out of 902 isolates originally collected, only 671 were identified as putative *E. coli* based on growth at 44.5 °C, ONPG hydrolysis, MUG hydrolysis, and IMViC response (Table 1). This probably reflects inexperience in selecting typical *E. coli* isolates from MFC agar plates with mixed colony types. Rejection of ONPG negative cultures may also have been too rigid a criterion for identification. Olsen et al. (13) report that for a variety of reasons, including culture shock, coliforms may not initially hydrolyze ONPG.

Of the 671 putative *E. coli* isolates only 17 (2.5%) were MUG negative (Table 1). Even fewer (0.4%) had an indole negative phenotype. All MUG negative bacteria were positively identified as *E. coli* isolates by their substrate utilization pattern on Biolog plates. None had substrate utilization patterns characteristic of other environmental species of *Escherichia* (14). There were no

Table 1. MUG negative *Escherichia coli* frequency in groundwater samples from 10 Kentucky counties.

County	Total isolates tested	Putative *E. coli*	MUG negative *E. coli*	Indole negative *E. coli*
Bourbon	192	145	1	2
Daviess	30	24	0	0
Fleming	123	86	3	1
Hickman	21	19	1	0
Hopkins	40	28	0	0
Logan	12	11	0	0
Russell	109	84	5	0
Shelby	68	43	1	0
Todd	100	79	2	0
Woodford	207	152	4	0
Total	902	671	17	3

Table 2. Effect of sonication on fluorescence in MUG negative *Escherichia coli* environmental isolates (fluorescence in three samples).

Isolate	Initial MUG response	Unsonicated MUG response	Sonicated MUG response
ATCC 25922	+	+ + +	+ + +
121B	−	− − −	− − −
432A	−	− − −	− − −
516A	+	+ + +	+ + +
533A	+	+ + +	+ + +
622A	−	− − −	− − −
622B	+	+ + +	+ + +
622D	−	− − −	− − −
622E	−	− − −	− − −
815B	−	− − −	− − −
834C	−	− − −	+ − −
834E	−	− − −	+ + −

water samples from which only MUG negative *E. coli* were isolated.

Glucuronic acid is a substrate used in the Biolog plates, and all MUG negative *E. coli* in our samples were able to utilize this substrate. With the exception of isolates 834C and 834E, sonicated MUG negative *E. coli* did not hydrolyze MUG in washed cell assays, indicating that impermeability was not the cause of the MUG negative phenotype in most of our isolates (Table 2).

DISCUSSION

Our results agree with those of Rice et al. (15), which suggest that the natural frequency of MUG negative *E. coli* is quite low. The frequency of MUG negative *E. coli* in individual counties ranged from 0 to 6% but this distribution was not statistically significant based on a chi-square test.

In a survey of published reports on MUG negative *E. coli*, Chang et al. (4) noted one case in which 8.3% of the environmental *E. coli* isolates were MUG negative. Shadix and Rice (16) also found a slightly higher frequency of MUG negative isolates than we did. Their samples came from sites with potentially more recent fecal contamination. If MUG positive *E. coli* survive longer in the environment than MUG negative *E. coli*, a higher frequency of MUG negative *E. coli* would be expected in freshly contaminated sites. This may explain some of the differences between the results for different environmental samples. Further studies are needed to establish whether environmental or cultural factors affect the survival and hence the frequency of MUG negative *E. coli*.

Using the MUG test as the sole criterion for *E. coli* enumeration in environmental samples, such as ours, should not seriously underestimate fecal contamination simply because MUG negative *E. coli* are in groundwater. Clearly, they are, but their numbers are low, and MUG negative *E. coli* were never the sole *E. coli* isolates obtained from an individual water sample. A presence–absence test for *E. coli* would not be misleading on that basis alone.

An entirely different question is whether the MUG-based tests have an unacceptably high rate of false negative samples that contain MUG positive *E. coli*. Defined-substrate tests compare favorably with membrane filtration assessments of fecal coliforms in untreated water (6,13) and some treated water (12). They also compare favorably with EPA-approved MUG-based tests (7); however, several studies (6,7,13) found numerous false negative samples using commercial defined-substrate media to monitor *E. coli* in treated water. The MUG positive *E. coli* were recovered from the false negative water samples. For that reason, further studies are needed to address the efficacy of defined-substrate tests for treated and untreated water.

Whether the MUG negative phenotype represents failure to express β-glucuronidase protein or inability to take up MUG remains an issue. All MUG negative isolates we examined were able to hydrolyze glucuronic acid but not MUG. Olsen et al. (13) suggest that injury, impermeability, lack of gene expression, or nonuse may all account for the MUG negative phenotype in *E. coli*. Clark et al. (6) and Covert et al. (6) showed that some MUG negative isolates regained the MUG positive phenotype upon further culture. Rice et al. (15) indicate that β-glucuronidase activity can be induced in virtually all *E. coli* isolates. Bej et al. (2,3) showed that MUG negative *E. coli* may contain genes for β-glucuronidase (*uid* A). The MUG negative phenotype in our isolates, however, was persistent.

Glucuronide is transported into *E. coli* by a permease (10). One mechanism that could cause the MUG negative phenotype would be failure of the permease to transport MUG across the cell membrane. Our results suggest that when our isolates were made permeable to MUG by sonicating them, in all but two cases, it had no effect on MUG hydrolysis by MUG negative bacteria. That the MUG positive phenotype was restored after sonication in two of the isolates suggests that multiple reasons for the MUG negative phenotype exist. We have not further investigated the specific reason for the MUG negative phenotype in our isolates.

Commercially prepared media such as Colilert, which incorporate defined-substrate technology (ONPG-MUG) to rapidly identify and enumerate fecal contamination of groundwater, could be useful tests to monitor groundwater contamination from agricultural sources if the issue of false negative samples is resolved. Colitag is an experimental medium that is not EPA-approved for the analysis of drinking water. Although, its formulation and incubation conditions are more demanding than the Colilert test, it could be an effective media for enumerating fecal coliforms in instances where MUG negative *E. coli* are suspected. Colitag incorporates tryptophane as a substrate to induce characteristic indole formation in *E. coli*. Although indole negative isolates were found, in 671

independent tests, we did not observe any cultures that were both MUG negative and indole negative. The addition of an indole test permits a rapid and convenient physiological check on MUG negative isolates.

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