Accelerated Detection of Microbes Utilizing an Organic Particle Catalyst in the Total Coliforms and *Escherichia coli* MMO-MUG (Colilert®) test

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Additional information is available at the end of the chapter

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1. Introduction

In 1992, the United States Environmental Protection Agency (EPA) approved the first dual total coliform and *Escherichia coli* (*E. coli*) direct detection test for public drinking water [1]. The MMO-MUG test, commercially known as Colilert® (Idexx Laboratories Inc., Westbrook, ME), could detect 1 total coliform or 1 *E. coli* in a 100 mL sample in 24 hours of incubation [2]. Over the next decades, the original MMO-MUG and variants have achieved worldwide use with consistently robust accuracy. Recently one form of the MMO-MUG, Colilert-18®, achieved European Union recognition as an ISO standard for total coliforms and *E. coli* [3].

One barrier to the utilization of new methods has been the need for an 18 to 24 hour incubation time. One form of the MMO-MUG, Colilert-18®, has a shorter incubation time than the original but requires an inconvenient and time consuming pre-heating step. Employing a new strategy that fosters the development of biofilm by incorporation of inert natural particles in the sample was reported to significantly reduce the incubation time of the MMO-MUG drinking water tests and other microbiological analyses.

The natural particles (see Figure 1) are made as a dried hydrophilic colloid extract obtained from *Gelidium cartilagineum*, *Gracilaria confervoides*, *Pterocladia lucida* and related algae of the class *Rhodophyceae*. These particles (Colloidands®, Pilots Point LLC, Sarasota, FL) have no innate significant nutritive value with the following composition: total fat 0%, saturated fat 0%, polyunsaturated fat 0%, monounsaturated fat 0%, cholesterol 0% sodium <1 mg/L, potassium 23 mg/L; total carbohydrate 0.7 g/L, fiber 0.1 g/L, sugar 0 g/L, protein 0 g/L, iron 10 g/L.

The particles are a heterogeneous natural mixture:
Figure 1. High resolution photomicrograph of the phycocolloid particles (Colloidands®, Pilots Point LLC, Sarasota, FL)

A study was conducted with the original MMO-MUG (Colilert®) to determine if from cold water < 8°C the incorporation of the particles could significantly reduce detection time of total coliforms and \textit{E. coli}.

2. Materials and methods

2.1. Activity of the particles

In order to establish that the particles were acting as a physical catalyst, the Colisure® variation of the MMO-MUG test was used. This test uses a yellow/gold beta-galactosidase substrate that becomes red/magenta when positive. Accordingly, the exact physical location of the beginning of the development of color could be directly determined photographically.

Colloidands® particles (Pilots Point LLC, Sarasota, FL) were added to the Colisure® test at 10 grams per liter. Both the standard Idexx \textit{Klebsiella pneumoniae} (\textit{K. pneumoniae}) and \textit{Escherichia coli} (\textit{E. coli}) quality control bacteria were utilized. Photographic records were taken each hour of incubation at 35°C for 20 hours at a concentration of 10 bacteria per mL.

2.2. Level of sensitivity

2.2.1. Quality control \textit{Klebsiella} and \textit{Escherichia coli} (Idexx Laboratories Inc., Westbrook, Maine)

Figure 2 and Figure 3 describe the complete protocol by which the MMO-MUG (Colilert®) was examined for its ability to detect 1 total coliform and 1 \textit{E. coli} in 16 hours. For the purpose of
studying universally available bacteria, the standard quality control strains available from the manufacturer of Colilert® (Idexx Laboratories Inc., Westbrook, ME) were utilized. Virtually all water testing laboratories use these for quality control or have access to them.

![Diagram of test protocol](image)

Figure 2. Test Protocol Part I for the detection of 1 total coliform Klebsiella and 1 E. coli in 16 hours

Analysis was made both by visual observation and also by an instrument. Visual observation: Yellow color development (for total coliforms and E. coli) and fluorescence (E. coli) was determined each hour while there was a person in the laboratory. The observations were structured to best represent the optimum work pattern in the standard drinking water laboratory: inoculation no later than 4 pm (1600 hours) and the reading of results at 8 am (0800 hours) the next day.

Instrumented Analysis: Figure 4 presents the Pilots Point Monitor (Pilots Point LLC, Sarasota, FL) that was used. This instrument utilizes white light sent through a sample and determines the change in three parameters: Luminosity, or white to black (a measure of turbidity), called the “L” value; a change from red to green (called the “a” value), and a change from blue to yellow (called the “b” value). See Figure 5. Measurements are made every 15 minutes. The instrument can measure light changes from 365 nm through the visible spectrum therefore it can detect both the color change produced by total coliforms and the fluorescence produced by E. coli.
2.3. Isolates from source water

Lake source water from the supply to the Regional Water Authority (New Haven, CT) was obtained. While protected from human intrusion, there are abundant animal life, particular deer, rabbit, and small animals. This is the same water source that was used in the original certification of the MMO-MUG test [4].
The same protocol as for the quality control bacteria described in the protocol (see Figure 2 and Figure 3) was used. To avoid possible enhancement of enzyme stimulation by the substrates present in the MMO-MUG formula, the source water samples were processed by the classical membrane filtration method as described in Standard Methods [5]. Bacteria consistent with total coliforms and *E. coli* were identified to species (API® ID strip range, bioMérieux, Durham, NC).

3. Results

3.1. Activity of the particles

Figure 6 presents the results of the Colloidands® particle analysis. The top tube is inoculated with Idexx Klebsiella, the bottom tube with Idexx *E. coli*. The original color of the Colisure® is yellow/gold; a positive occurs when the hydrolysable substrate is cleaved during growth and multiplication. The particles have largely sunk to the bottom of the tube. It is clearly shown that the red/magenta color is overwhelmingly seen in the particle layer at the bottom of the test tube. Over time, the positive color spreads fully into the liquid layer. The observation that the particle layer consistently is where the color develops first substantiates the ability of the particles to accelerate the growth and multiplication of the target bacteria.

3.2. Level of sensitivity

Table 1 and Graph 1 present the visual observation of the Colilert® test with and without phycocolloid particles at low numbers of *E. coli*, Table 2 and Graph 2 present the visual
observation of Colilert® with Enterobacter cloacae (E. cloacae) added, Table 3 and Graph 3 present the observation with K. pneumoniae added, and Table 4 and Graph 4 present the observation with E. coli added. The water was < 8°C and there were 7 grams of Colloidands® particles per liter of Colilert®. Overall the presence of the particles decreases the time to a positive, both for K. pneumoniae and E. coli demonstrating the efficacy of the particles. Specifically both for the Idexx Klebsiella and Idexx E. coli, Colilert® is able to detect 1 bacterium in 100 mL of water in 16 hours or less as seen in Figure 7.

| E. coli added count | Hours to Yellow (no particles) | Hours to Fluorescence (no particles) | Hours to Yellow (7g particles) | Hours to Fluorescence (7g particles) |
|---------------------|-------------------------------|-------------------------------------|-----------------------------|-------------------------------------|
| 10 CFU (test a)     | 16                            | 16                                  | 10                         | 10                                  |
| 10 CFU (test b)     | 19                            | 22                                  | 11                         | 15                                  |
| 10 CFU + NTO (test a) | 13                          | 13                                  | 11                         | 11                                  |
| 10 CFU + NTO (test b) | 13                          | 13                                  | 10                         | 10                                  |
| 1 CFU + NTO (test a)   | 14                          | 16                                  | 11                         | 11                                  |
| 1 CFU + NTO (test b)   | 14                          | 15                                  | 11                         | 10                                  |

Table 1. Colilert® in Cold Water < 8°C

Graph 1. Comparison of Time to Detection of MMO-MUG (Colilert®) with and without phycocolloids. CFU (colony forming units), NTO (non-target organisms), Neg (negative)
Graph 2. Comparison of Time to Detection of MMO-MUG (Colilert®) with and without phycoccolloids with *E. cloacae* added. CFU (colony forming units), NTO (non-target organisms), Neg (negative)

### Table 2. Colilert® in Cold Water < 8°C with *E. cloacae*

| E. cloacae (24 isolates) | Hours to Yellow (no particles) | Hours to Fluorescence (no particles) | Hours to Yellow (7g particles) | Hours to Fluorescence (7g particles) |
|--------------------------|---------------------------------|--------------------------------------|---------------------------------|--------------------------------------|
| 10 CFU (test a)          | 16                              | Neg                                  | 12                              | Neg                                  |
| 10 CFU (test b)          | 18                              | Neg                                  | 11                              | Neg                                  |
| 10 CFU + NTO (test a)    | 13                              | Neg                                  | 12                              | Neg                                  |
| 10 CFU + NTO (test b)    | 13                              | Neg                                  | 12                              | Neg                                  |
| 1 CFU + NTO (test a)     | 21                              | Neg                                  | 15                              | Neg                                  |
| 1 CFU + NTO (test b)     | 20                              | Neg                                  | 15                              | Neg                                  |

Graph 3. Comparison of Time to Detection of MMO-MUG (Colilert®) with and without phycoccolloids with *K. pneumoniae* added. CFU (colony forming units), NTO (non-target organisms), Neg (negative)

### Table 3. Colilert® in Cold Water < 8°C with *K. pneumoniae*

| K. pneumoniae (31 isolates) | Hours to Yellow (no particles) | Hours to Fluorescence (no particles) | Hours to Yellow (7g particles) | Hours to Fluorescence (7g particles) |
|-----------------------------|---------------------------------|--------------------------------------|---------------------------------|--------------------------------------|
| 10 CFU (test a)             | 16                              | Neg                                  | 10                              | Neg                                  |
| 10 CFU (test b)             | 19                              | Neg                                  | 11                              | Neg                                  |
| 10 CFU + NTO (test a)       | 13                              | Neg                                  | 11                              | Neg                                  |
| 10 CFU + NTO (test b)       | 13                              | Neg                                  | 10                              | Neg                                  |
| 1 CFU + NTO (test a)        | 21                              | Neg                                  | 15                              | Neg                                  |
| 1 CFU + NTO (test b)        | 20                              | Neg                                  | 14                              | Neg                                  |
Figure 7 presents the actual pictures of the replicates of the analysis of the Colilert® test to detect 1 bacterium per 100 mL. As can be seen, each of the 100 mL samples was clearly positive. As shown in Figure 6, the particle layer at the bottom of the water collection vessel is densely colored. The liquid is also clearly positive.

Figure 8 and Figure 9 present the results of the testing shown in Figure 7 as follows: from the 100 mL samples in Figure 7, 1 mL of supernatant was added to a 13 x 100 mm polystyrene test tube and placed in the PPM instrument. Readings in the PPM instrument were taken and a definite change in the “L”, “a”, and “b” values can be seen which indicate positive results for both quality control Idexx E. coli and Idexx total coliform Klebsiella. Because the light path in the test tube is much smaller than in the 100 mL vessel, the time required for a positive in a 100 mL sample is longer.

In all configurations – 1 mL in a test tube and 100 mL in a water collection vessel – the phycocolloids significantly decreases the time to detect the quality control Idexx total coliform Klebsiella and the Idexx quality control E. coli. In addition, the phycocolloids decreases the time to detect 1 bacterium in 100 mL of cold water < 8°C to within 16 hours.
Figure 7. Replicate Testing with Idexx Quality Control E. coli and Klebsiella showing the ability of Colilert® to detect 1 bacterium per 100 mL within 16 hours in a cold water <8°C sample with phycocolloids added.

Figure 8. PPM Readings of 10 Quality Control E. coli at the 1 CFU/100 mL level.
Figure 10 and Figure 11 present 1 mL duplicate data from the PPM instrument on detection of 10 bacteria per mL of Idexx E. coli in Colilert® with the addition of phycocolloids in cold water < 8°C. The “b” value on the graph indicates the color change from colorless (Colilert® negative) to yellow (Colilert® positive). Once the yellow color starts to develop, a strong yellow color is observed in a two hour time period. In the experiment shown in Figure 10, the time to detection was 8.5 hours. In the experiment shown in Figure 11, the time to detection was 9.5 hours. The phycocolloid particles not only reduces the time to positive detection but also reduces the time bacteria spend in lag phase and increases the multiplication of bacteria in log phase as seen in the graphs.
Figure 10. Actual PPM Data for detection of Idexx E. coli at 10 CFU/mL. Positive detection at 8.5hr incubation.

Figure 11. Actual PPM Data for detection of Idexx E. coli at 10 CFU/mL. Positive detection at 9hr incubation.
4. Discussion

Since the introduction of the MMO-MUG (Colilert®) test in 1989 for the simultaneous detection of total coliforms and *E. coli* from drinking water, wastewater, bathing water, and other uses, attempts to decrease the time to a positive have been met with limited success. A variation of the Colilert® test, Colilert-18®, reduced the time from 24 hours to 18 hours incubation but requires the heating of the water sample adding an additional hour or two. Water samples are transported to the laboratory chilled by regulation. However, because many water samples arrive and are processed at the end of the work day after collection, the time to a result is often in the middle of the work day, thus delaying remediation and response and adversely affecting the public’s health protection.

The two types of bacteria that generally exist are described as either sessile (a unit that attaches to a surface or exist in a biofilm) or planktonic (existing freely in bulk solution). Antonie van Leuwenhoek described biofilms in 1674 as “animalcules” through observation of material scraped from human tooth surfaces with his microscope, but with advances in technology, biofilms can more accurately be described [6]. Biofilms can be described as microbial communities that are sessile and grow on surfaces surrounded by a matrix of extracellular polymeric substances; microcolonies are distinct communities of bacterial cells of one or many different species that are surrounded by a matrix. The advantages of biofilm formation by bacteria is that it provides protection against antibiotics, disinfectants, and environments that are constantly changing [7]. The key to the catalyst activity of the phycocolloids is their ability to interact with the bacteria and the attendant production of microcolonies. The bacteria multiply much faster when attached to the particles as microcolony biofilm.

The micro particles increase the surface area in the liquid broth and allow microbes that multiply in vitro to establish a biofilm. In effect, the micro particles act in an analogous way as a catalyst does in a chemical reaction. In the microbiology area, the micro particles provide multiple attachment surfaces for the microbes to "establish residence". Microbes prefer surfaces on which to grow and multiply rather than being free in a liquid environment. For example, the microbes may experience quorum sensing, which accelerates the generation of a biofilm. The biofilm is produced when the microbes multiply, and it yields colonies of microbes that are held together by external capsules, pili, and glycocalyxes of the microbes which, in the broad context, are surface components, such as polysaccharides, proteins and/or mixtures thereof. The micro particles are static, in that they are not consumed but serve as a physical structure that provides shelter and attachment and promotes the multiplication and expression of the target microbe. There may be attached nutritive elements on the micro particles that serve to stimulate the development of the bacterial nidus. The micro particles may be colloidal, in suspension, or a combination. Any materials or structures that encourage the growth of microbes on a biofilm are highly preferred for use in this invention. Supporting the catalytic activity of the phycocolloids is the observation that growth starts first at the bottom of the test tubes, where particles have gravitationally settled and microbial biofilms have developed, attached to the particles. Additional particles are distributed with the remainder of the admixture, but have not yet been associated with metabolizable substrate creating a color
change. The image of the test tubes illustrates well how the particles expedite the detection of the bacteria (i.e., *E. coli*) targeted by the metabolizable substrate of the test mixture, and the increased sensitivity associated therewith; i.e., the micro particles provide surface area or attachment surfaces for the *E. coli* microbes to establish residence, grow, and multiply (e.g., creating biofilms), which growth is indicated by the color change created when the nutrient portions of the metabolizable substrate are metabolized by the microbes.

This report demonstrates that phycocolloids introduced into the classical Colilert® formula significantly decreases the time to a positive. By visual observation with both standard quality control *Klebsiella* and *E. coli*, plus with total coliforms and *E. coli* isolated from source water, it was demonstrated that Colilert® could detect 1 target bacterium in 100 mL of cold water < 8°C in repetitive experiments. The detection in cold water < 8°C is particularly important because the Colilert-18® variation, while rated positive in 18 hours, requires the pre-heating of the water sample before the test is started, thus effectively increase the time to approximately 20 hours.

The 16 hour benchmark is particularly important for laboratory work flow. It provides the ability to perform a 4 to 8 test – in by 4 pm, finished by 8 am, the optimum for the work flow. Further enhancing the time to detection is the use of the PPM instrument. With it, detection of 1 total coliform and 1 *E. coli* in cold water < 8°C was decreased by several hours; furthermore the instrument signals a positive at the time it is positive.

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