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METHOD OF MAKING BACTERIOLOGICAL DETERMINATIONS

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This invention relates to a rapid method for making qualitative and quantitative bacteriological determinations and more particularly to a rapid method for making bacteriological determinations which makes use of radioisotopes and devices for detecting radioactivity.

One of the paramount problems in the field of public health is caused by the time lapse required before bacteriological tests can be completed. In controlling the quality of public water supplies, for instance, 48 to 72 hours are required before results of bacteriological examinations can be obtained by the standard method in use as described in "Standard Methods for the Examination of Water and Sewage," American Public Health Association, American Water Works Association, Federation of Sewage and Industrial Wastes Associations, 10th edition, 1955, henceforth referred to as "Standard Method." During that time waterborne disease could be spread throughout a community. Similar delays in obtaining bacteriological data necessary for quality control or other important ends occur in the milk and food industries, sterilizing industry, clinical medicine, and other areas. An object of this invention is to reduce by a large factor the elapsed time required for bacteriological determinations and to provide a simpler method of analysis.

The method consists of preparing inoculum from samples containing or suspected of containing known or unknown kinds and/or quantities of bacteria. The inoculum is then inoculated into a general or specific bacteriological medium containing one or more ingredients in radioactive form and placed under incubation conditions. The radioisotope is thus metabolized by any bacteria present of the type that would respond to the medium. The detection of radioactivity in the bacteria or their metabolic products demonstrates the presence of the organisms. The level of radioactivity detected as a function of time is proportional to the numbers of organisms. Tests for radioactivity are made at short intervals until a conclusive level of activity or lack of it is demonstrated.

A specific application of this method that demonstrates its practicality is the development of a rapid presumptive method for the early detection of coliform organisms. This group of organisms was selected because it is the most important group in sanitary science. It was also selected because the method would use C^{14} which has a low specific radioactivity and success with it would indicate that many other bacteriological tests using the same or higher specific activity isotopes would be practical.

The Standard Method uniformly adopted for the presumptive test for coliform organisms in raw or treated water supplies is based on the ability of coliform organisms to ferment lactose with the production of gas, a large portion of which is CO_2 .

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The specific test used to demonstrate the new method is as follows:

Sterile 0.5 percent lactose broth is prepared according to said Standard Method except that the lactose used is composed totally or partially of a C^{14} substituted lactose, such as 1- C^{14} lactose. A portion of the water sample to be tested is inoculated directly into the lactose broth, or the bacteria in any quantity of sample may be concentrated by filtration or centrifugation and then inoculated into the broth. The broth is incubated and is aerated constantly, intermittently, or just prior to making a determination. The gas expelled from the culture vessel by the aeration is allowed to escape through a filler, pad or membrane previously immersed in $Ba(OH)_2$. The carbon dioxide present in the exhaust gas is trapped as barium carbonate. The entrapment object is then dried and counted for radioactivity. Coliform organisms, if present in the culture, split the radioactive lactose, releasing radioactive $C^{14}O_2$, which is trapped as $BaC^{14}O_3$. Significant radioactivity (in the order of two times the background radiation) constitutes completion of the presumptive test for coliform organisms in the sample. The level of radioactivity evolved by the culture with respect to time is directly proportional to the number of organisms present in the test portion. Generally, the test can be accomplished in one or two hours or less. Little, if any, bacterial reproduction is required because the method is sensitive enough to detect the metabolism of a very small number of cells as compared to the 1.7×10^8 cells per ml. required for the evolution of visible gas. Metabolism occurs at radiodetectable rates in the lag phase as well as in the growth phases, thus eliminating the lag phase delay common to all present bacteriological identification methods.

This method was used on known concentrations of pure cultures of *Escherichia coli* and to test for coliform organisms in raw river water samples of unknown bacteriological quality. It was successful in each case, detecting as few as 26 organisms in a period of one hour as confirmed by replicates run by the Standard Method procedure.

The following are some of the experiments that have been performed. They have been selected to show the development, present status, and various aspects of the method of making bacteriological determinations described herein.

Example I

This experiment was performed to determine earliest detectability of $C^{14}O_2$ generated by *E. coli* in standard lactose broth to which 0.02 mg. lactose 1- C^{14} (1.79 microcuries/mg.) per 100 ml. of medium has been added. One ml. of a suspension of *E. coli* of 0.15 optical density (10^4 - 10^5 organisms) was inoculated into 5 of 6 tubes containing 5 ml. of the lactose broth. All tubes were incubated at 37° C. under aeration. The exhaust gas of each tube was diffused into a corresponding tube containing saturated solutions of $Ba(OH)_2$. The $Ba(OH)_2$ tubes connected to the 5 inoculated tubes were removed singly after 1, 2, 4, 6 and 8 hours of incubation. The contents were filtered through membrane filters to remove any $BaCO_3$. The filter membranes were dried and measured for radioactivity. The sixth tube was a control and was filtered, and the filtrant counted at the beginning and end of the experiment.

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TABLE 1

Time (hrs.)	Radioactivity (Counts per minute above background)	
	Increment	Cumulative
0 control	12	
1	85	85
2	99	184
4	228	412
6	522	934
8	390	1,324
8 control	30	

Example II

This experiment was run to determine earliest detectability of $C^{14}O_2$ generated by *E. coli* in standard lactose broth of which total lactose content (0.5%) is 1- C^{14} lactose (1.79 microcuries/mg.). One 10 ml. portion of the 1- C^{14} lactose broth was inoculated with approximately 26 *E. coli* (as determined by plate count of inoculum suspension). The tube culture was incubated at 37° C. under constant aeration. The exhaust gas from the culture was diffused into a saturated solution of $Ba(OH)_2$. The tube containing the $Ba(OH)_2$ was removed hourly, and the contents filtered through a membrane filter. The filter membranes were dried and measured for radioactivity.

TABLE 2

Time (hrs.)	Cumulative Radioactivity (Counts per minute above background) ¹	
	Increment	Cumulative
1	9	9
2	23	32
3	197	229
4	79	308
5	25	333
6	313	646
7	2,291	2,937
8	3,431	6,368

¹ Background=29 counts per minute.
² Point of presumptive determination.

Example III

Much of the radioactive $BaCO_3$ was remaining in solution and passing through the filter. The following method was tried in an attempt to retain all $BaCO_3$ produced. One 10 ml. portion of 1- C^{14} lactose broth (same as in Example II) was inoculated with less than 100 *E. coli* (by dilution). The culture was incubated at 37° C. under constant aeration. Into the glass tubing carrying the exhaust gas a cotton plug impregnated with saturated $Ba(OH)_2$ solution was inserted. The plug was replaced with a fresh one at the end of an hour, and the exposed plug was dried, cut into portions and measured for radioactivity. After one hour of incubation, the first plug, cut into four parts, yielded a total activity of 5167 counts per minute. Another plug exposed during the second hour and measured in two parts yielded a total activity of 1506 counts per minute. Control plugs run subsequently, and measured at the end of 1, 2 and 3 hours gave counts of 418, 112 and 36 counts per minute respectively. Paper fiber pads moistened with a saturated solution of $Ba(OH)_2$ may be used in place of the impregnated cotton plugs.

Example IV

This example demonstrates the rapid presumptive coliform test. A 10 ml. portion of the lactose broth used in Examples II and III was heat-sterilized and divided into two .5 ml. portions. One portion was inoculated with approximately 125 *E. coli* (plate count). Both portions were incubated at 37° C. under constant aeration. The

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gas from each was exhausted through disks of absorbent paper impregnated with a saturated solution of $Ba(OH)_2$. The disks were replaced with fresh ones each hour. The exposed disks were dried and measured for radioactivity.

TABLE 3

Time (hrs.)	Radioactivity (Counts per minute above background) ¹			
	Test		Control	
	Increment	Cumulative	Increment	Cumulative
0	106		99	
1	172	272	67	67
2	309	481	38	105
3	1,154	1,635	36	141
4	4,075	5,710	36	177
5	12,579	18,289	27	204

¹ Background=21 counts per minute.
² Point of presumptive determination.

Example V

This example demonstrates the rapid presumptive coliform test. Water was taken from the Potomac at downtown Washington and brought to the laboratory where 5-10 ml., 5-1.0 ml. and 5-0.1 ml. portions were inoculated into standard (nonradioactive) lactose broth. An equal volume, 55.5 ml., of the same sample was filtered through a filter membrane. The filter membrane was removed and immersed in a test tube containing five ml. of 1- C^{14} standard lactose broth. A sterile control was run with this tube. All tubes were incubated at 37° C. The two tubes containing the 1- C^{14} lactose broth were aerated. The carbon dioxide in the exhaust gas from them was collected by the pad method. At short intervals, the pads were replaced with fresh ones, and the exposed pads dried and counted for radioactivity. The 15 tubes run by the Standard Method were examined for gas periodically. The first pad was removed after 30 minutes and demonstrated a radioactivity far in excess of that needed for a positive determination. The results of the tests are shown below in Table 4. After ten hours of observation, no gas had yet appeared in the Standard Method tubes. The tubes were then incubated overnight, and upon examination 24 hours after inoculation, gas was seen in all tubes. The ability of the radioisotope method to reduce significantly the time required to determine the presumptive presence of coliform organisms has thus been demonstrated experimentally. Theoretical calculations based on respiration rates of *E. coli* in standard lactose broth and the specific activity of C^{14} indicate that less than 20 cells can be detected in one hour with the present counting equipment. This assumes no bacteria multiplication during that period, and is not materially affected by whether the organisms are in lag or growth phase.

TABLE 4

Presumptive tests of raw Potomac River water
(Radioisotope Method.)

Time (mins.)	Radioactivity (Counts per minute above background) ¹			
	Test		Control	
	Increment	Cumulative	Increment	Cumulative
30	1,598	1,598	28	28
60	1,578	3,176	23	51
95	2,428	5,604	18	69

¹ Background=22 counts per minute.
² Point of presumptive determination.

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Example VI

This example demonstrates the use of the method in confirming the presence of coliform organisms. It is similar to the method for the presumptive coliform test described in Examples I through V except that the lactose broth used also contains an ingredient such as brilliant green bile or crystal violet which selectively promotes the growth of coliform organisms. The organisms in question are placed in the confirmatory medium. Determinations are made as described in the cited examples.

Example VII

This example demonstrates the use of the test for determination of the total bacteria count. A sterile, synthetic nutrient broth was prepared as follows by adding 0.1 g. of $MgCl_2$, 6 g. of Na_2HPO_4 , 3 g. of KH_2PO_4 , 5 g. of $NaCl$, 1 g. of NH_4Cl , 8 milligrams of $Na_2S^{35}O_4$, 100 ml. of a 4% aqueous glucose solution to 900 ml. distilled water. Thus the only source of sulfur, which is vital to bacterial growth, was labelled. Fifty ml. of raw Potomac River water was filtered through a membrane filter, the filter rinsed with 10 ml. of distilled water, and the membrane immediately immersed in 250 ml. of the above described broth. The culture was aerated continuously and incubated at 37° C. At intervals, 5 ml. of the culture were withdrawn, filtered through a membrane filter, and the filter rinsed with 10 ml. of distilled water. The membrane was then removed, dried and counted for radioactivity. Bacteria would incorporate the sulfur causing successive filter membranes to increase in radioactivity.

TABLE 5

Time (hrs.):	Radioactivity, counts per minute
1-----	1190
2-----	1340
3-----	1650
24-----	3510

Example VIII

This example demonstrates use of the test in clinical medicine for the rapid presumptive diagnosis of coliform infections of the bloodstream such as are associated with wounds, peritoneal exudates and perforated ulcers. One ml. of the suspected blood is placed in 10 ml. of lactose broth containing a C^{14} substituted lactose similar to that described in Example II. The culture is incubated and aerated, and exhaust carbon dioxide is trapped, dried and counted as described in Example II. When the cumulated radioactivity of the dried pads becomes significantly higher than the background, the determination is considered positive.

Example IX

This example demonstrates use of the method in clinical medicine for the rapid presumptive diagnosis of coliform infections of the urinary tract. Twenty-five ml. of the suspected urine is filtered through a membrane filter. The filter membrane is placed in a C^{14} substituted lactose broth as described in Example II and the determination made as described in that example.

Example X

This example demonstrates use of the method in clinical medicine to determine the antibiotic of choice to be used against unidentified infectious bacteria. A routine blood culture is made from a sample of the patient's blood. The bacteria to be tested are transferred from the culture into 10 ml. of trypticase soy broth (17.0 g. trypticase, 3.0 g. phytone, 5.0 g. sodium chloride, 2.5 g. dipotassium phosphate, 2.5 g. glucose and 1,000 ml. distilled water), and incubated 6 hours. Suspensions made from the resulting culture are inoculated into 2 sets of 5 tubes each containing 2 ml. of trypticase soy broth in which a C^{14} substituted glucose is used. Two ml. dilutions containing 100 micrograms per ml. of the antibiotic

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to be tried are added to one set of tubes. All tubes are incubated at 37° C. The contents of one tube of each set are removed hourly, filtered through membrane filters, and the filters rinsed with 50 ml. distilled water. The membranes are then removed, dried and counted for radioactivity. If the radioactivity of the membranes through which the cultures containing the antibiotic is filtered is significantly lower than that of the corresponding membranes through which the cultures with no antibiotic is filtered, the antibiotic is shown to be effective against the organism.

Among the advantages of this invention over the Standard Method is the great time saving factor which permits bacteriological results to be obtained in time for close quality control of water supplies. A means to permit bacteriological monitoring of drinking water has long been sought, but never before attained. This development promises to be a highly significant one in the control of waterborne disease. The test is simpler than the Standard Method procedure. The fact that the radioactivity collected is directly proportional to the number of organisms allows a quantitative determination with a single test portion. The Standard Method relies on a statistical method to determine the most probable number of organisms present in the sample. The latter method requires the inoculation, incubation, and observation of from 5 to 15 test portions for each quantitative determination. The new method results in a considerable saving of labor and materials. The Standard Method requires dilutions to be made from the original sample in order to obtain quantitative results. Frequently the correct range of dilutions is missed, since it must be estimated. When this happens the test becomes inconclusive or, at best, qualitative only. The new method is sensitive to low or high concentrations of organisms and no dilution procedures are required. Radioactive means of gas detection for bacterial identification and enumeration are used in the new method rather than the visual means which are used in the Standard Method for the detection of coliform organisms. This is a new and far more sensitive qualitative and quantitative bacteriological tool.

The time-saving factor mentioned above is equally important in other bacteriological determinations made by this method. In the milk industry the method enables rapid bacteriological quality determinations of the raw milk, finished milk products, and throughout all stages of production. The same is true in the food preparation and packing industry. The method enables close study of disinfection and sterilization processes for the determination of speed, mechanisms and other important factors of various agents and methods. Likewise, it enables beneficial study of industrial and laboratory bacterial culturing methods and agents. It has an important application in the field of clinical medicine where it enables more rapid identification of infectious bacteria. With such knowledge, the physician may prescribe the therapeutic agent of choice. At present he is faced with the choice of waiting several days for bacteriological identification during which time the infection may become worse, or of administering a broad spectrum preparation which may or may not combat the pathogens. Radioactive rather than visual means are used for qualitative and quantitative bacteriological examinations. In addition to the time-saving factor, this innovation makes the new method tremendously more sensitive and more accurate than other known methods.

The above-description and examples are intended to be illustrative only and not to limit the scope of the invention. Any variation or departure therefrom which conforms to the spirit of the invention is intended to be included within the scope of the appended claims.

I claim:

1. A method of determining the presumptive presence of the coliform group of bacteria in a material selected from the group consisting of water, foods, and body

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fluids, which method comprises inoculating a sample of the material to be tested or of organisms removed from the material into a lactose broth containing a C^{14} substituted lactose, incubating said broth, and measuring the radioactivity of the respiratory $C^{14}O_2$ evolved.

2. A process as recited in claim 1 in which the respiratory $C^{14}O_2$ evolved is absorbed in barium hydroxide thereby forming $BaC^{14}O_3$, and the radioactivity of the formed $BaC^{14}O_3$ is then measured.

3. A method of confirming the presence of coliform organisms in a material selected from the group consisting of water, foods, and body fluids, which method comprises placing the organisms in question into a coliform selective growth broth containing a C^{14} substituted lactose,

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incubating said broth, and measuring the radioactivity of the respiratory $C^{14}O_2$ evolved.

4. A process as recited in claim 3 in which the respiratory $C^{14}O_2$ evolved is absorbed in barium hydroxide thereby forming $BaC^{14}O_3$, and the radioactivity of the formed $BaC^{14}O_3$ is then measured.

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