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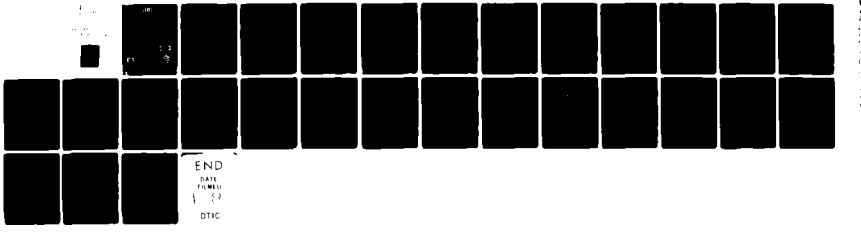

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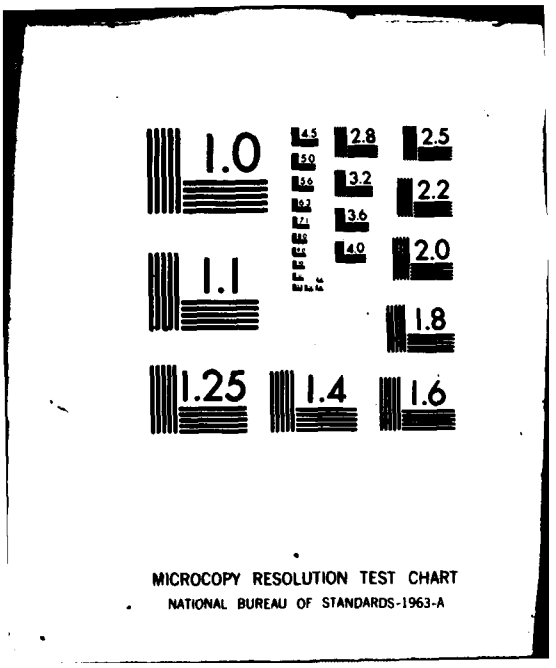
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CHEMICAL SYSTEMS LABORATORY CONTRACTOR REPORT
ARCSL-CR-80064

KIT TESTS FOR RAPID DETECTION OF VIABLE BACTERIA AND VIRUSES

Final Report
by
R.H. Moyer

October 1980

GEOMET Technologies, Inc.
Gaithersburg, Maryland 20760

Contract No. DAAK11-79-C-0063

US ARMY ARMAMENT RESEARCH AND DEVELOPMENT COMMAND
Chemical Systems Laboratory
Aberdeen Proving Ground, Maryland 21010

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER ARCSL-CR-80064	2. GOVT ACCESSION NO. AD-A091796	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) KIT TESTS FOR RAPID DETECTION OF VIABLE BACTERIA AND VIRUSES		5. TYPE OF REPORT & PERIOD COVERED Contractor Report June 1979 - June 1980
		6. PERFORMING ORG. REPORT NUMBER GTI REPORT NO. LF-830
7. AUTHOR(s) R. H. Moyer		8. CONTRACT OR GRANT NUMBER(s) DAAK11-79-C-0063
9. PERFORMING ORGANIZATION NAME AND ADDRESS GEOMET Technologies, Inc. Gaithersburg, Maryland 20760		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Commander/Director, Chemical Systems Laboratory ATTN: DRDAR-CLJ-R Aberdeen Proving Ground, MD 21010		12. REPORT DATE October 1980 13. NUMBER OF PAGES 29
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) Commander/Director, Chemical Systems Laboratory ATTN: DRDAR-CLC-B Aberdeen Proving Ground, MD 21010		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE NA

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Detection	Kit test	Bacteria detection
Kit bacteria	Rapid detection	Virus detection
Viruses	p-Iodonitrotetrazolium	Viable bacteria
Field kit	Nucleic acid	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Metabolism-linked reagents for reduction of p-iodonitrotetrazolium (INT) were optimized and reaction conditions for detecting viable bacteria on the surface of a filter were defined. The test detected 10^4 - 10^5 viable bacteria within 30 minutes.

The feasibility of detecting nucleic acids and bacteriophage by binding N-Methyphenazinium methosulfate (PMS) to the nucleic acid followed by catalytic reduction of nitroblue tetrazolium (WBT) was demonstrated. The sensitivity of the test was 10^{-9} gm of RNA or DNA, corresponding to 10^7 - 10^8 virions.

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PREFACE

The work described in this report was authorized under Contract Number DAAK11-79-C-0063. This work was started in June 1979 and completed in June 1980. The experimental data are recorded under Number 79-04-011.

The use of trade names in this report does not constitute an official endorsement or approval of the use of such commercial hardware or software. This report may not be cited for purposes of advertisement.

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KIT TESTS FOR RAPID DETECTION OF VIABLE BACTERIA AND VIRUSES

1. INTRODUCTION AND SUMMARY

This is the final report on Contract DAAK11-79-C-0063, which was entitled "Kit Tests for Rapid Detection of Viable Bacteria and Viruses." The contract had two major objectives:

(a) Explore biochemical approaches to select candidate detection methods for bacteria, optimize reagents and reaction conditions for several species of bacteria, and initiate design of self-contained kits for field use.

(b) Study feasibility of nucleic acid binding of N-Methylphenazinium methosulfate (PMS) for detection of viruses, optimize reagents and reaction conditions and evaluate the feasibility of adapting the method to kits for field use.

Most of the work on the test for bacteria was carried out using a filtration assembly developed by Chemical Systems Laboratory (CSL) and a reagent mixture based on reduction of p-iodonitrotetrazolium (INT). Earlier work by CSL had identified a reagent mixture suitable for use with INT, and much of the work on the contract was an extension of the CSL work. Autoreduction of INT in the course of a test was a persistent difficulty, but a reagent mixture stable for more than 60 minutes has been developed. The INT test can detect approximately 10^5 viable organisms in 30 minutes. This general sensitivity was obtained with several organisms. Some work was also done on optimization of two reagent mixtures based on reduction of resazurin. These may prove useful for distinction between some classes of organisms.

The basic requirements for a field kit for detection of bacteria were identified, and the conditions necessary for preparation of stable lyophilized reagents were defined. The major requirements for the filter portion of the field kit are that it should be a single-part, disposable unit in which the deposition area is visible without the need for disassembly.

The feasibility of detecting nucleic acids and bacteriophage by means of binding PMS and subsequent reaction with a reduced pyridine nucleotide and an indicator was demonstrated. However, tests with live virus have not been completed. The sensitivity of the test is 10^{9}-10^{-10} gm of nucleic acid, which corresponds to approximately 10^7 virions. Test sensitivity should be improved to the extent that 10^5 virions can be detected.

2. METHODS AND MATERIALS

All of the work on the test for bacteria, and a large portion of that on the test for virus, was carried out using a filtration assembly developed

by Chemical Systems Laboratory (CSL). Reagents and test materials were either purchased commercially or supplied by CSL. The filtration assembly is briefly described in paragraph 2.1, and the most important reagents used are listed in paragraph 2.2. Reagent formulations used for individual tests are identified in paragraph 3 as part of the description of the tests.

2.1 Filtration Assembly. The assembly consists of three lucite discs (70-mm diameter) stacked on top of each other and bolted together. The discs contain six coincident holes located at uniform intervals in an annular arrangement. A 47-mm filter placed on the lower disc rests on six recessed 13-mm support screens. The holes in the central disc are conical and serve as reaction wells. The upper disc contains six hollow cylindrical projections (2.36-mm ID) that fit through the cones in the central disc and press on the filter. Metal Luer fittings are sealed into the upper portions of these projections.

For use, a 47-mm filter is placed on the lower disc, and the assembly is bolted together. Bacterial suspensions are filtered manually under pressure from a syringe. The upper disc is removed, reagents are placed in the conical wells in the central section and bacterial metabolic reactions take place on the surface of the filter.

2.2 Reagents and Materials. Glass-distilled H_2O was used for preparation of all reagent solutions. The reagents generally used throughout the program were:

Phosphate-buffered Saline (PBS) - The following salts were dissolved in 1000 ml H_2O then filtered through Gelman, Acropor AN 200 (0.2 micron).

NaCl	8.5 gm
K_2HPO_4	8.6 gm
KH_2PO_4	2.4 gm
Tween 80	0.5 gm
pH	7.2

Schaedler broth (Difco 0534 - 17) - The dehydrated powder (2.84 gm) suspended in 100 ml H_2O, heated to boiling for 1-2 minutes, cooled and filtered through Gelman, Acropor AN 200 (0.2 micron).

Adenosine - 5'-triphosphate (ATP) (Calbiochem) - The disodium salt was dissolved in 0.1N 3-(Morpholino)propanesulfonic acid (MOPS) (Calbiochem) and adjusted to pH 6.8.

Reduced Nicotinamide-adenine dinucleotide (NADH) - The disodium salt was dissolved in 0.1N tris(hydroxymethyl)aminomethane (TRIS) (Calbiochem) and adjusted to pH 7.5.

Resazurin (MCB, CRZ-8) - Stock solutions, containing 2 mg/ml resazurin were prepared in 20 mg/ml bovine serum albumin (Calbiochem, Fraction V, B grade) and filtered through Gelman Acropor AN 200 (0.2 micron). The indicator was diluted as necessary immediately before use.

N-methylphenazinium methosulfate (PMS) (Calbiochem) - Stock solutions of 2.5 mg/ml PMS were prepared in distilled H_2O. They were protected from light and diluted as necessary immediately before use.

p-Iodonitrotetrazolium (INT) (Baker, Y094 - Stock solutions of 1.5 mg/ml INT were prepared in either 50 mg/ml bovine serum albumin (Calbiochem, Fraction V, B grade) or dextran (MW, 40,000, ICN Pharmaceuticals, Inc.). The solutions were filtered through Gelman Acropor AN 200 (0.2 micron), protected from light, and diluted as necessary immediately before use. The INT solutions used early in the program were prepared in bovine serum albumin. These solutions proved unsatisfactory because of autoreduction of INT. Solutions prepared in dextran were much more stable and were used throughout the last half of the program.

Deoxyribonucleic Acid (DNA) (Calf Thymus, A grade, Calbiochem) - Solutions were freshly filtered PBS and diluted in either PBS or H_2O.

Ribonucleic Acid (RNA) (Yeast, A grade, Calbiochem - Solutions were prepared in freshly filtered PBS and diluted in either PBS or H_2O.

Filters - All filters used in the filtration assembly were 47 mm in diameter. The materials used in the test for bacteria were:

Gelman, Metricel GA6 (0.45 micron pore size)
Gelman, Metricel GA8 (0.22 micron pore size)
Nuclepore (0.4 micron pore size).

The filter finally selected for the virus test was Millipore PHUP (0.5 micron pore size). This material is very thin, flexible, unbacked Teflon.

3. TECHNICAL RESULTS

The major objectives of the program were the development of kit tests for rapid detection of viable bacteria and evaluation of the feasibility of developing similar kits for detection of viruses. Technical results obtained in meeting these objectives are described below.

3.1 Test for Bacteria. The filtration assembly described in paragraph 2.1 was used for all of the work on the test for bacteria. A reagent system based on reduction of p-iodonitrotetrazolium (INT) was used for most of

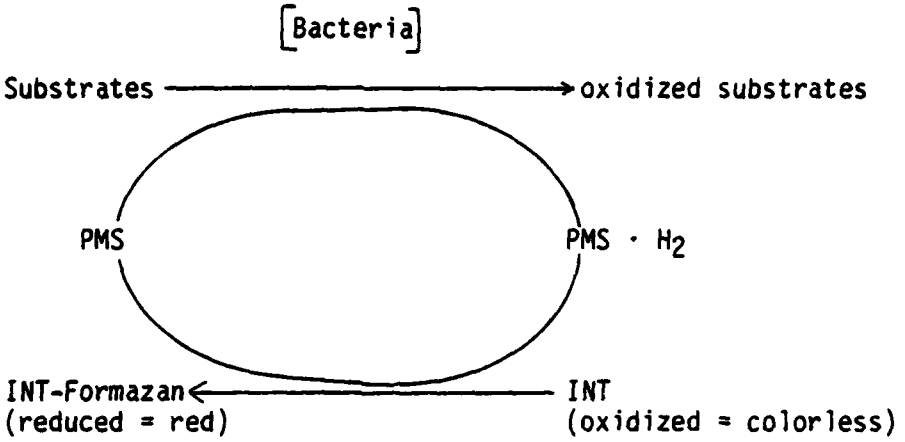
this work. Limited tests of a reagent system based on reduction of resazurin produced results equivalent to those obtained with INT. All tests were conducted at room temperature, and 30 minutes was arbitrarily set as the maximum time permitted for a test.

3.1.1 INT Test. Tetrazolium salts, such as INT, have been widely used in histochemistry for visualization of dehydrogenase activity. They are particularly useful in this application because they are converted from colorless, water-soluble compounds to brightly colored insoluble precipitates on reduction. The reduced form remains at the reduction site and does not diffuse back into the medium. This property is also useful in a test for bacterial metabolism carried out on the surface of a filter. In the case of INT, the initial reagent is essentially colorless; and, the red, insoluble formazan produced on reduction remains where it was formed, in contact with the bacteria on the filter surface.

A number of substrates, including glucose, glutamate, pyruvate, lactate and citrate were tested in an effort to maximize sensitivity. Best results were obtained with a mixture containing 10 mg/ml glucose and 10 mg/ml glutamate in PBS adjusted to pH 7.3. Previous work, carried out by CSL, had shown that INT reduction rates by bacteria are significantly accelerated in reaction mixtures containing Schaedler broth. A number of combinations of glutamate/glucose/ATP/NAD/ with Schaedler broth and Schaedler broth alone were tested. Most rapid reduction of INT by E. coli was obtained with the following reaction mixture:

Substrate	Reaction Concentration
glutamate	7.5 mg/ml
glucose	7.5 mg/ml
Schaedler Broth (Difco)	10%
K_2HPO_4/KH_2PO_4	7.5 mg/ml
pH	7.25
Electron Carrier	
N-Methylphenazinium methosulfate (PMS)	0.1 mg/ml
Indicator	
p-Iodonitrotetrazolium (INT)	0.5 mg/ml

The overall reaction sequence is:



Typical results obtained with this reaction mixture and E. coli from a 24 hour culture on solid media were:

Organisms (Total per test)	Observation
0	no obvious change in 30 minutes
5×10^4	trace color in 30 minutes
2×10^5	trace color in 17 minutes, distinct in 30 minutes
5×10^5	trace color in 5 minutes, dark in 17 minutes
5×10^6	trace color in 1 minute, dark in 5 minutes

Results comparable to these were obtained routinely with fresh cultures. Sensitivity was highly dependent on the condition of the organisms. Fresh dilutions of young cultures were detected at levels of 5×10^4, while aged dilutions in H_2O or organisms from old refrigerated cultures could not be detected at levels of 1×10^5 in 30 minutes. This result is not surprising. Since detection depends on metabolism, any lag in achieving maximum respiration impacts directly on test sensitivity. Resting cells and cells from old cultures, though viable, have much slower metabolic rates than cells from cultures in the logarithmic growth phase.

3.1.1.1 Reaction Conditions. Three CSL filtration assemblies, having different deposition areas, were used on the program. Tests by both CSL and GTI consistently showed that sensitivity increased as the deposition area was decreased, suggesting that INT reduction by bacteria on a filter surface requires some minimum density of bacterial cells. The following approximate sensitivity limits for detection of E. coli were obtained in 30-minute tests in the three assemblies:

Assembly	Total cells for 30-minute detection	Deposition area	Cells/mm^2
0.070 inch	5×10^4	2.5 mm^2	2.0×10^4
0.093 inch	1×10^5	5.2 mm^2	1.9×10^4
5 mm	5×10^5	19.6 mm^2	2.5×10^4

The cell densities (cells/mm^2) necessary for detection are comparable in the three assemblies, suggesting that some factor, other than physical accumulation of a visible INT deposit, is limiting test sensitivity.

Reduced PMS is very readily oxidized by O_2, which competes with INT for electrons generated by the organisms. Autoxidation of PMS.H_2 produces H_2O_2 which accumulates because PMS completely inhibits catalase. Competition for O_2 in the reduction of INT and subsequent accumulation of H_2O_2 could be the major factor limiting test sensitivity.

Accumulation of H_2O_2 was prevented by including NaCN in the reagent mixtures. This produced a significant improvement in test sensitivity, as shown by the following comparison between reduction rates obtained from a regular reaction mixture and one which contained 0.25 mg/ml NaCN:

Reaction Mixture	Organisms/test	Reduction rate**
	0	>30 minutes
Regular INT*	5×10^4	30 minutes
	5×10^5	15 minutes
Regular INT	0	30 minutes
with 0.25 mg/ml	5×10^4	20 minutes
NaCN	5×10^5	8 minutes

*Reaction mixture described in paragraph 3.1.1.

**Time required for development of distinct red color on filter surface, as viewed through the unreacted reagent in the filtration well.

The above test was carried out with E. coli from a 24 hour/37°C culture on solid media. The cells were scraped from the media and diluted in H_2O immediately prior to the test. The filtration assembly was a CSL 0.093-inch unit containing a 0.45 micron Gelman GA-6 filter. The holes in the base of the assembly were stoppered after filtration of the organisms, prior to dispensing reagents in the wells. The assembly was shielded from light during the test.

Improved sensitivity, as indicated by the above observations, was routinely obtained by adding NaCN to the reagent mixtures. However, the addition of cyanide increased the rate of autoreduction of INT in proportion to the cyanide concentration. The concentration used in the above tests was the maximum that could be tolerated without obscuring the filter surface in the 30-minute test period. Dilute cyanide solutions are unstable and the compound cannot be lyophilized. It is therefore not feasible to include the NaCN modification in a reagent system for field use. However, the results of the NaCN tests indicate that competition from molecular O_2 and subsequent H_2O_2 accumulation limit the sensitivity of the INT test.

It is possible that H_2O_2 removal can be achieved by an indicator such as 2,6-dichlorophenol indophenol, whose oxidation and reduction are reversible. The results of two sets of tests conducted at the end of the program indicate that the addition of traces of this compound improves the sensitivity of the INT reagents. For these tests, an INT-dextran stock solution was prepared as described in Section 2.2, and microliter quantities of an aqueous solution of 2,6-dichlorophenol indophenol were added until the solution had a barely perceptible blue tint. This solution was mixed with PMS and substrates immediately before use. The reagent was tested in parallel with a reagent prepared from an aliquot of the same INT stock solution that contained no indophenol. The organism used for these tests was E. coli from a 24-hour culture, diluted in PBS. Results obtined were:

Blanks - Slight pink after 30 minutes and definite red color after 45 minutes with regular INT; completely clear in INT-indophenol after 45 minutes.

$2x10^4$ - Indistinguishable from blank at 30 and 45 minutes with regular INT; trace more color than blank at 30 minutes and definite positive at 45 minutes with INT-indophenol.

$2x10^5$ - Definite positive at 15 minutes with both reagents.

The major effect of the 2,6-dichlorophenol indophenol is elimination of INT autoreduction during the test. However, the organisms remained active for 45 minutes in the INT-indophenol reagent, suggesting that indophenol was scavenging H_2O_2. While the approach appears promising, more work will be needed to determine the extent to which sensitivity can be improved.

3.1.1.2 Reagents for CSL Tests. The reagents described in paragraph 3.1.1 were used for CSL tests. The substrate portion, containing glutamate, glucose, phosphate buffer and Schaedler broth, is completely stable. However, the INT and PMS are photosensitive and deteriorate rapidly, even in diffuse light. Lyophilization of the complete reaction mixture in a single vial resulted in a product that rehydrated readily and performed as well as the same mixture prior to lyophilization. However, the lyophilized mixture showed some deterioration after several days of accelerated aging at 37°C. Since the time remaining prior to the scheduled CSL tests was too short to allow adequate shelf-life testing, it was considered advisable to lyophilize the PMS, INT and substrates in separate vials. Reagent concentrations in the three vials, after rehydration in 2.0 ml H_2O were:

A - PMS	0.1 mg/ml
B - INT	0.5 mg/ml
BSA (Fraction V)	2.5 mg/ml
C - glutamate	7.5 mg/ml
glucose	7.5 mg/ml
K_2HOP_4/KH_2PO_4	7.5 mg/ml
Schaedler broth	10%
pH	7.25

Rehydration of the reagents in A and C was very rapid, but the INT/BSA mixture in B required up to 2 minutes for complete rehydration. For use, A was rehydrated in 2.0 ml H_2O, which was then transferred successively to B and C to produce the final reaction mixture in a volume of approximately 2 ml.

The lyophilization cycle used for preparation of the reagents delivered to CSL was 18 hours. This was too short, and the reagents contained enough residual moisture to cause severe deterioration during storage at room temperature for approximately 1 month. A set of reagents prepared using a 44-hour cycle showed no deterioration after 2 months of accelerated aging at 37°C. The 44-hour cycle appears adequate for preparation of reagents having satisfactory stability.

3.1.1.3 CSL Evaluation of INT Test. The utility of the INT reagent system for detection of viable bacteria was evaluated by CSL in several series of tests. These tests included:

Laboratory Tests. Tests to check the lyophilized reagents were conducted at CSL on delivery of the reagents. Test duration was limited to 30 minutes because autoreduction of INT began to obscure the deposition area at the end of this time. The following organisms were tested and detected at the levels indicated:

	Organisms per test
Escherichia coli	5×10^4
Enterobacteria aerogenes	2×10^4
Staphlococcus lactis	5×10^4
Bacillus globigii (BG) spores	2×10^4

The vegetative cells were obtained from 18-hour cultures in liquid media. The BG spores had been prepared and stored in gelatin - phosphate buffer. All organisms were serially diluted in H_2O prior to filtration. The sensitivity of the test for BG spores was highly dependent on the matrix in which the spores had been prepared and stored. Spores from stock suspensions in H_2O were very inactive, and 2×10^6 were needed to produce a faintly positive test.

Field Tests. The INT reagents were used with CSL filtration assemblies and 0.45 micron fibers to test background and aerosolized BG spores after collection by an XM2 biological sampler. In these tests, a positive result from fewer than 10^2 spores was considered a false positive, and a negative result from more than 10^2 was considered a false negative. The results of 77 tests showed 13% false positives and 17% false negatives. The sensitivity of the test is shown in Figure 1, in which the percent positive results is plotted against the logarithm of the number of spores per test.

Detection of 5×10^2 spores was accomplished with a reliability of 50%, but 7×10^5 was needed for 100% reliability. The INT used in these tests had been prepared in bovine serum albumin, and autoreduction during the 30-minute test period may have contributed to both the false positive and false negative results obtained. It was concluded that the test was sensitive enough to confirm an alarm but that sensitivity would need to be improved by a factor of 10^2 in order to confirm the absence of bacteria. It was also apparent that a satisfactory field kit would require a simpler filtration assembly and more stable reagents than those used in these tests.

Pathogen Tests. Sensitivity for detection of pathogenic organisms was measured by CSL. All of the organisms tested were detected, but the sensitivity for some was unsatisfactory. The poor sensitivity may have been due to the inability of the organisms to metabolize the substrates in the INT reaction mixture. However, a more probable cause is poisoning by H_2O_2 from autooxidation of PMS.H_2, as described in paragraph 3.1.1.1. In this case, a stable H_2O_2 scavenger compatible with the reaction system will need to be identified to achieve satisfactory sensitivity for detection of pathogens. It is possible that H_2O_2 removal can be achieved by the addition of a trace of 2,6-dichlorophenol indophenol or a similar compound, but testing of this approach was too limited for firm conclusions. (See paragraph 3.1.1.1.)

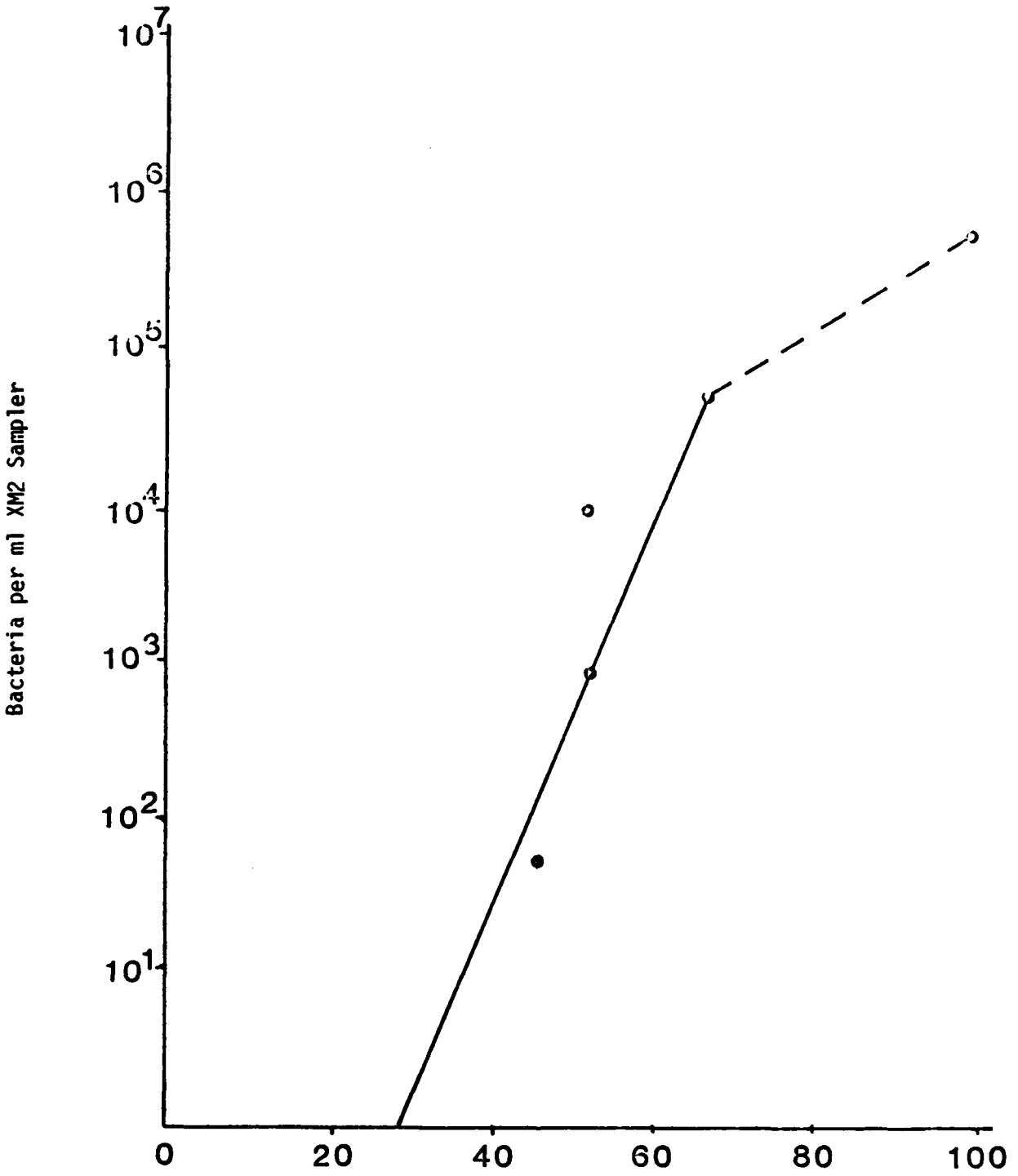


Figure 1 - Field Test Results

Background Tests. A number of positive results were obtained by CSL from 10^2 - 10^4 viable organisms collected from ambient air by the XM2 biological sampler. A positive test from 10^4 organisms is consistent with the sensitivity of the test, but 10^2 should not be detectable. In the CSL field tests, 5×10^2 BG spores were detected with a reliability of 50%, even though 5×10^4 from liquid suspensions were needed for positive tests. The apparent improvement in sensitivity obtained with the aerosolized spores could be due to increased metabolic activity induced by aerosolization. In the case of atmospheric background, a significant number of the organisms collected could have been metabolically active but incapable of replication on the media used for the viable counts. Additional work with test aerosols and atmospheric background will be needed before background interference can be evaluated.

3.1.1.4 Autoreduction of INT. Autoreduction of INT during the 30-minute test period was one of the most consistent difficulties encountered. The INT preparations used during the first half of the program, and for the CSL field tests, contained bovine serum albumin, which accelerated autoreduction. Substitution of dextran for the albumin produced a more stable reagent, but some autoreduction was still evident. Addition of a trace of 2,6-dichlorophenol indophenol to INT-dextran preparations (paragraph 3.1.1.1) appeared to produce a completely stable reagent. However, the indophenol approach was attempted late in the program, and the data obtained are insufficient for assurance that autoreduction difficulties have been resolved.

Positive blanks resulting from autoreduction of INT were overcome by modifying test conditions. The CSL field tests were conducted using an INT preparation that had a fast rate of autoreduction and produced positive blanks that could not be eliminated by prefiltration of the water or PBS. In these tests, sample solutions were filtered then the reagents were dispensed into the wells above the filter. The reagent drained through the filter in the course of the test and any INT-formazan formed by autoreduction of INT in the volume of reagent above the filter was deposited on the filter to produce a result identical to that from bacteria. The difficulty was much more pronounced in tests where PBS containing Tween 80 had been filtered than in those where H_2O had been used because the reagent drained more rapidly through filters that had contacted the surfactant. The difficulty was overcome by stoppering the holes in the base of the assembly prior to dispensing the reagent in the wells.

The scope of the problem and the effectiveness of the solution were demonstrated in tests where three ports of an assembly were stoppered and the other three left open. E. coli suspensions, obtained from a 24-hour culture on solid media, were diluted in H_2O and filtered to produce the following filter loadings:

Filter Port	Organisms (Total per test)	
1	0	Holes in filter base left open
2	1×10^5	
3	5×10^5	
4	0	Holes in filter base stoppered after filtration of organisms
5	1×10^5	
6	5×10^5	

An INT-reaction mixture, containing all the reagents required for the test, was prepared and left exposed to room light for approximately 30 minutes. At the end of this time, the solution showed significant autoreduction but was still transparent. The six wells were filled with the partially reduced solution, requiring approximately 0.2 ml per well. After 10-minutes incubation, wells 1 through 3 showed definitie reduction, with the color intensity graded as predicted from the number of organisms present. After 30 minutes, spots 1 through 3 were very red and showed little difference between the blank and two tests. Essentially all of the reagent had drained from these three wells during the 30 minutes. In the stoppered ports, spot 6, containing 5×10^5 organisms, showed distinct color after 10 minutes and was dark red after 30 minutes. Spot 5 showed definite reduction at 30 minutes, while spot 4, the blank, remained clear. After incubation for an additional 30 minutes (total time = 60 minutes) there was still no red deposit on spot 4.

These observations clearly implicated drainage of autoreduced reagent through the filter as the major cause of the positive blanks. They also established that a significantly autoreduced reagent could detect bacteria and not show a blank reaction if drainage through the filter was prevented.

Results equivalent to those obtained by stoppering the reagent wells were obtained by inverting the assembly and applying the reagents to the support screens on the downstream side of the filter. The assembly remained inverted during the 30-minute test period. Any reduced INT initially present in the reagent or formed by autoreduction in the wells was retained on the downstream side of the filter. Only freshly filtered reagent, draining through the filter, contacted the bacteria on the upstream side.

Inverted incubation was operationally more convenient than stoppering the wells, but observation of the test prior to the end of the 30-minute period was difficult. Both modifications of the test procedure accommodated INT autoreduction. However, minimization or elimination of autoreduction would be preferable.

3.1.2 Resazurin Test for Bacteria. Some exploratory studies of reaction systems based on reduction of resazurin were carried out prior to selection of the INT reagent system used throughout most of the program. Most of these studies were done before the CSL assemblies were available, using tests carried out in capillary tubes. Reagents were mixed in a droplet on Parafilm and approximately 30 µl of the mixture was used to completely fill a 2.5 cm length of capillary tubing. The organism used was an unidentified strain of E. coli scraped from a refrigerated nutrient agar slant and suspended in MOPS buffer, pH 6.8. The tests were qualitative, and a viable count was considered unnecessary. Typically, the suspensions were diluted until no turbidity was evident to the unaided eye. Final reaction mixtures, in the capillary tubes, were estimated to contain 10^5 to 10^6 organisms per test. All buffers, indicators and reagent solutions were filtered through Gelman, Acropor AN-200 (0.2 µ) immediately prior to use. All tests were carried out at room temperature.

3.1.2.1 Substrate/Electron Carrier/Indicator The most sensitive reaction mixture tested was:

Reagent	Final Concentration (mg/ml)
Glucose	10
ATP	3
Resazurin	0.3
PMS	0.1

Capillary tubes, containing 10^5 to 10^6 organisms per tube showed visible reductions after 10 to 15 minutes, were fully red by 30 minutes, and had decolorized centers in 45 minutes. The ends of the tubes remained red due to air oxidation of resorufin. Control tubes, with no added bacteria, showed no visible reduction after several hours. Inclusion of NAD in the reaction mixture did not accelerate the rate of reduction of the indicator. However, omission of ATP slowed the reduction rate, and approximately 60 minutes was required for visible resazurin reduction by the organisms.

The role of ATP in accelerating reduction is not obvious. It cannot penetrate cell membranes but could possibly activate permeases to facilitate glucose uptake. Addition of hexokinase to a mixture containing ATP and glucose produces the following reaction:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{Hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

When a reaction mixture containing ATP and glucose was held at room temperature for 15 minutes following addition of hexokinase, resazurin reduction by the organisms was equivalent to that observed when ATP was omitted. This observation indicates that ATP acceleration cannot be replaced by glucose-6-phosphate or ADP.

Other substrates tested included glutamate, oxalacetate, lactate, pyruvate and citrate. None of these appeared to offer any advantage over glucose. However, ATP was not included in the tests carried out with these substrates, and the tests will need to be repeated in the presence of ATP in an effort to identify combinations superior to ATP/glucose.

A limited number of tests were conducted with the ATP-glucose-resazurin reagents in the CSL filtration assemblies. Results were generally unsatisfactory because resorufin, the reduced form of resazurin, is soluble and diffuses away from the reaction zone. Loss of reduced indicator could be partially controlled by using 10-mm discs of filter material in place of the 47-mm filters used for the INT test. Two tests of the ATP-glucose-resazurin reagents in a CSL assembly with 10-mm filter discs were conducted at CSL late in the program. The organisms used for these tests were fresh cultures of E. coli and Staphlococcus lactis diluted in H_2O.

The sensitivity for detection of E. coli was not as good as that obtained earlier in capillary tubes. In complete reaction mixtures, containing PMS, resazurin reduction was observed in 15 minutes with 4×10^7 organisms and in 30 minutes with 4×10^5. In a parallel test using a reagent mixture without PMS, more than 160 minutes was required for reduction by 4×10^7 organisms. The sensitivity for detection of S. lactis was 5-10 minutes for 1×10^5 and approximately 60 minutes for 1×10^3 organisms. Omission of PMS from reaction mixtures did not significantly decrease reduction rates. The very large difference in reduction rates between the two organisms in this test is surprising, since they were essentially equivalent when checked earlier with the INT test (paragraph 3.1.1.3). Use of selective reactivity in different tests could prove to be a useful approach for discrimination between organisms or between pathogens and background.

3.1.2.2 NADH/Indicator. A reaction system containing only NADH and an indicator relys on bacterial flavoproteins to mediate reduction of the indicator by NADH. For example, a mixture of NADH, resazurin and bacteria should produce the following reaction:

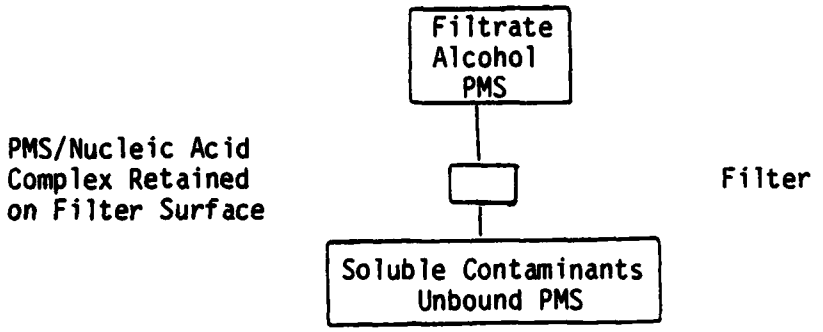
$$\text{NADH} + \text{Resazurin} \xrightarrow{\text{Bacteria}} \text{NAD} + \text{Resorufin}$$

The simplicity of this approach is appealing, and encouraging results had been obtained in screening tests with Serratia marcescens. Attempts to repeat the tests with E. coli produced disappointing results. The approach was abandoned because E. coli appeared to be totally devoid of Diaphorase activity needed to mediate resazurin reduction by NADH. However, the results obtained at CSL using the ATP-glucose-resazurin reagents with E. coli and S. lactis (paragraph 3.1.2.1) suggest that the reaction may be valuable

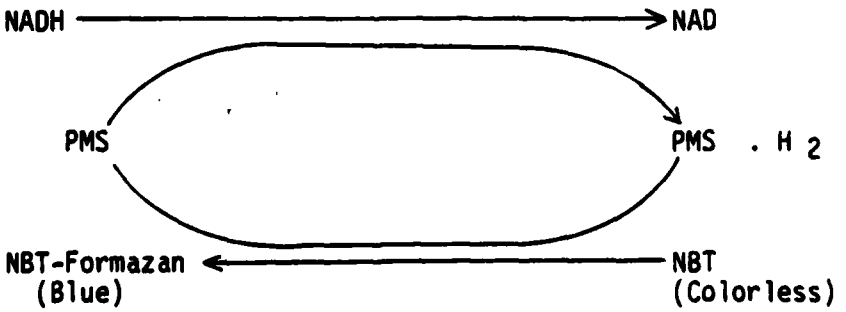
for distinction between organisms. Reduction of resazurin by E. coli required the electron carrier, PMS, while reduction by S. lactis did not. This observation suggests that S. lactis has a high level of Diaphorase activity and could be easily detected with an NADH-indicator agent. Pathogenic organisms of interest should be screened with this system because the sensitivity for detection of some of them could be good and they could be detected without interference from organisms like E. coli.

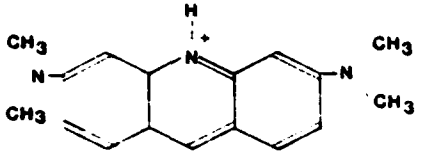
3.2 Virus Test. The test for virus is based on formation of complexes between PMS and viral nucleic acid. Compounds like PMS and the Acridine dyes that consist of three coplanar-fused aromatic rings form strong complexes with nucleic acids. The similarity between PMS and Acridine orange and an illustration of the types of complexes formed are shown in Figure 2. The complexes with native DNA contain approximately one dye molecule per six base pairs. In denatured or single-stranded nucleic acids, like soluble RNA, the ratio of dye to nucleic acid is much higher.

The test for virus is carried out on the filtrate from the test for bacteria after precipitating viral nucleic acid by mixing the filtrate with alcohol containing PMS:

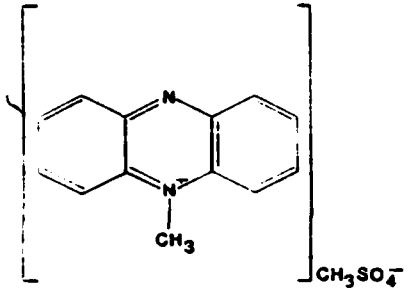


The indicator reaction is carried out on the filter surface after washing PMS from the PMS/nucleic acid complex. In the subsequent indicator reaction, the PMS in the complex serves as a catalyst to transfer electrons from NADH to NBT, producing blue NBT-formazan.



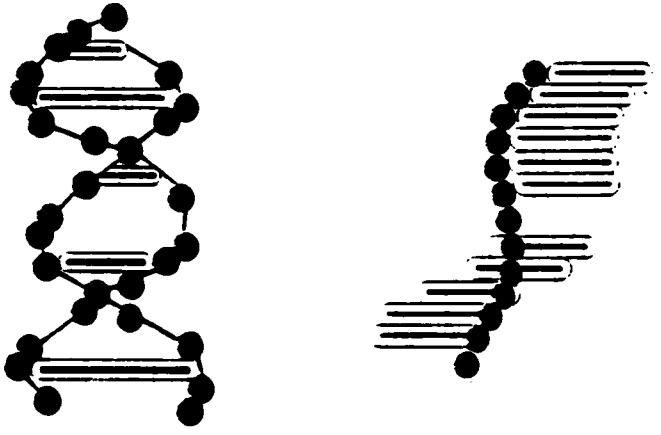


ACRIDINE ORANGE



PHENAZINE METHOSULFATE

NUCLEIC ACID STAINS



DNA COMPLEX

RNA COMPLEX

NUCLEIC ACID / PMS COMPLEXES

Figure 2. Nucleic acid stains and PMS complexes

3.2.1 Syringe-Filtration Test for Virus. The major difficulty in the virus test is reduction of the indicator by residual PMS that is not bound to nucleic acid. Most of the work on the program was directed toward development of a technique suitable for CSL tests of live material. The major portion of the effort was on techniques that employed syringes for manual filtration of the alcoholic PMS and wash solutions. In the course of this work, it became apparent that any technique in which a sample is pumped through a filter under pressure from a syringe requires some type of inlet to couple the syringe to the filter. The inlet is one major problem area that makes the syringe technique unsatisfactory for the virus test. Residual PMS, deposited in the inlet during filtration of the initial PMS solution, is picked up by the wash solution, leaving traces of PMS on the filter after washing. The sensitivity of the indicator reaction to PMS is so high that residues from the inlet can cause a false positive reaction, even after repeated washing.

A technique that utilized disposable pipet tips to prevent PMS contamination of assembly inlets during filtration of the PMS solution showed some promise in bench tests but was too unwieldy for use in a glove box. Typically, PMS was spilled or splashed onto the gloves and assembly at some point during a test series; gloves and equipment had to be cleaned before another test was attempted. The need for frequent cleanup is only a time-consuming nuisance in a glove box, but a PMS spill would terminate any test with live material. The syringe technique was therefore considered unsatisfactory for CSL tests and was replaced by vacuum filtration.

3.2.2 Vacuum-Filtration Test for Virus. A vacuum-filtration setup suitable for testing live material was assembled using the base of a 5-mm CSL filtration assembly, a vacuum flask, and the bottom of a Gelman 47-mm filter funnel. The base of the CSL assembly, complete with its six 13-mm support screens covering the deposition areas, was sealed onto the funnel bottom using the rubber sleeve supplied with the funnel. Bolt holes in the CSL assembly were stoppered, and vacuum applied to the flask was coupled only to the deposition zones. A 47-mm disc of Parafilm, having 2-mm holes coincident with the deposition zones, was placed over the assembly. A 47-mm Millipore FHUP filter (0.5 μ pore size) was placed over the Parafilm. Vacuum applied to the flask drew the FHUP filter into contact with the support screens, producing shallow wells at the deposition zones. The following sequence of manipulations was required to carry out a test:

- Prepare 96% (w/w) methanol solution by adding 3.0 ml H_2O to 90 ml anhydrous reagent grade methanol (3.0 gm H_2O + 72 gm methanol).
- Add 0.1 ml of 2 mg/ml aqueous PMS solution to 0.5 ml sample.
- Add 1.0 ml 96% methanol to PMS/sample mixture. 21

- Place FHUP filter on filtration apparatus and apply vacuum.
- Wet deposition zone with 96% methanol solution; this solution wets only the area of the filter in contact with the support screen, which remains wet for approximately 2 minutes.
- Filter alcoholic PMS/sample mixture prepared earlier; filtration must be started before deposition zone has dried; if zone has dried, wet it again with 96% methanol solution.
- Wash deposition zone with approximately 1.5 ml 96% methanol, applied in sequential drops.
- Draw air till deposition zone is dry.
- Place one drop of reagent mixture on deposition zone; reagent is prepared immediately before use by mixing one volume of 2 mg/ml NADH solution with one volume of 1 mg/ml NBT; reagent remains on surface of deposition zone and does not penetrate the filter, even with vacuum still on; vacuum may be left on or turned off during test.
- Blue color developes at a rate proportional to the quantity of nucleic acid present; blanks show little or no color in 30 minutes; 10^{-9} gm of DNA or RNA produces a definite color in less than 30 minutes.

The manipulations described may seem complex and unsuitable for a field kit. However, the objective was to adapt the technique to testing live material and demonstrate feasibility for virus detection. Tests at GTI and at the CSL laboratory indicated that the test sequence described could be carried out in a glove box and would be suitable for testing live virus. Simplified filtration assemblies and ancillary equipment will need to be developed for a field kit.

The sensitivity of the test was 10^{-9} grams of RNA or DNA, corresponding to 10^{7} to 10^{8} virions. In the CSL laboratory tests, 10^{8} bacteriophage were readily detected. However, the sensitivity of the test will need to be improved to the extent that 10^{-12} grams of nucleic acid, corresponding to 10^{4} to 10^{5} virions, can be detected. Sensitivity can be improved by increasing the PMS and NADH concentrations. The blank reaction from residual, unbound PMS increases rapidly with increasing PMS and NADH concentration and appears to be the main factor limiting sensitivity. The blank reaction

in the technique described was very low, and higher reagent concentrations can be used. Previous tests with a similar technique detected 10^{-11} grams RNA, and improvement to detection of 10^{-12} grams should be feasible.

Two features of the technique are critical for satisfactory performance:

Filter material--Major sources of contamination by PMS are lateral diffusion from the area adjacent to the deposition zone and back-diffusion from the downstream side of the filter. Repeated washing is ineffective in removing this contamination, and the only practical solution is use of a filter material like Teflon that is so hydrophobic that it is not wetted by 50-60% methanol. The 50-60% alcoholic PMS solution only penetrates the filter at the prewetted deposition zone, leaving no lateral residue to contaminate the subsequent test. After washing, air is drawn through the deposition zone until the zone is dry. The drop of reagent, subsequently placed on the zone, remains entirely on the filter surface; and, back-diffusion of PMS from the downstream side is impossible. Laterial diffusion can be controlled with filter materials like Nuclepore, but these are hydrophillic, and the reagent penetrates the filter. Back-diffusion of PMS from the downstream side during the test produces a false positive reaction.

Wash Solution--Methanol, ethanol and ispropanol have all been used for precipitation of PMS/nucleic acid and as wash solutions for removal of unbound PMS from the precipitate. To eliminate back-diffusion of PMS from the downstream side of the filter, it is necessary that the deposition zone be dried by drawing air through it before the NADH/NBT reagent is applied. Methanol has a higher vapor pressure than the other alcohols and dries more quickly. Anhydrous methanol is unsatisfactory as a wash solution because it wets all areas of the filter that it contacts. However, 96% (w/w) methanol wets only the area under vacuum, which is in contact with the support screens. This is also the only area penetrated by the PMS solution, and the wash step effectively removes unbound PMS.

These features of the test are critical for design and development of field kits. The need for drying the deposition zone before application of the indicator reagents dictates a requirement for an external vacuum. A simple filtration assembly, with vacuum supplied by a battery-powered personal monitor pump, should be satisfactory for field use. However, a number of breadboard assemblies will need to be constructed and tested before development of prototype units.

3.2.3 CSL Virus Tests. Lyophilized reagents for the virus test were supplied to CSL, and the virus test was included in the CSL field tests (paragraph 3.1.1.3). No meaningful results were obtained because the tests were carried out using manual filtration with CSL assemblies and Nuclepore filters. Work since these tests were conducted has demonstrated that manual filtration cannot be used, even under ideal laboratory conditions, and that Nuclepore filters are unsuitable for the test. These constraints were not known at the time and the field tests were conducted under conditions that precluded obtaining meaningful results.

Some tests of live virus have been conducted by CSL, using the vacuum filtration apparatus and technique described in paragraph 3.2.2. The objective of these tests was definition of the sensitivity of the test for detection of live virus and measurement of the quantity of virus lost by filtration. Results of the tests were inconclusive. The virus stock was in a medium containing 5% fetal calf serum, which appeared to be the main factor causing conflicting results. Suspensions of 10^7 - 10^8 units/ml were usually not detected, while 10^5 - 10^7 units may have been detected. In subsequent bench-top tests, DNA and RNA were added to the medium containing 5% fetal calf serum. The nucleic acids were detected more readily as the calf serum was diluted out, indicating that the serum inhibited the test.

None of the obvious explanations for the inhibition is completely plausible. Nucleases in the serum could have degraded the nucleic acids to the extent that they were not precipitated by the alcohol. This explanation is improbable because the viral protein coat should not have been removed prior to addition of alcohol. The alcohol should have precipitated the nucleic acid, allowing no time for nuclease activity to influence the test. There was enough protein present to occlude the PMS-nucleic acid complex and prevent contact with the NADH-NBT reagents. This possibility is being checked in bench-top tests using guanidine hydrochloride and urea to dissolve possible protein shields covering the PMS nucleic acid complexes. A satisfactory approach for overcoming the protein interference has not yet been identified, and the live virus tests have been temporarily interrupted.

4. CONCLUSIONS AND RECOMMENDATIONS

As a result of the work completed on Contract DAAK-11-79-C-0063, conclusions can be drawn, and recommendations can be made in the following areas:

INT Reagents--The reagents in the INT test for bacteria have been stabilized enough for use in field kits, but lyophilization time must be at least 44 hours. Autoreduction of INT during a test has been controlled enough for tests to be run for more than 60 minutes. However, a 30-minute test is recommended for field use. Except for some pathogens and BG spores that had been stored in H_2O, the sensitivity of a 30-minute test is approximately 10^5 organisms.

Trace quantities of 2,6-dichlorophenol indophenol in the reagent retards autoreduction of INT and appears to improve test sensitivity. It is recommended that the pathogen tests be repeated with a reagent containing indophenol to determine whether or not the poor sensitivity for some pathogens was due to accumulation of H_2O_2 during the earlier tests.

Filtration Assembly--The multiport CSL assemblies are indispensable research tools for developing the tests and optimizing reagents. However, the volume of sample that will be available in practical use is approximately 1 ml. Such a small sample can be tested in a single filter port. It would be desirable to have the deposition area visible in the assembled device, avoiding the need for partial disassembly in order to view the test. It is recommended that single-use, single-part, disposable filters, in which the desposition area is visible in the assembled unit, be developed for field use.

Background Interference--The limited number of background tests conducted by CSL indicate that the XM2 biological sampler can collect enough metabolically active biological material from outside air in a 40-minute period to produce a positive INT test. In some cases, enough viable bacteria were collected to produce a positive test, but in others, there should not have been enough. The background tests were too limited to determine whether or not the positive tests resulted from live bacteria incapable of growth under the conditions used for the viable counts or from mold spores, pollen grains, yeast and plant debris. It is recommended that more extensive background tests be conducted to define the cause of positive reactions not attributable to viable bacteria. These tests should include reagent combinations based on more than just the INT test.

Other Tests for Bacteria--Two observations made during tests of the ATP-glucose-resazurin reagents at CSL are particularly important. When PMS was included in the reaction mixture, resazurin was reduced by 10^3 S. lactis under conditions that required 10^5 E. coli. When PMS was omitted, the sensitivity for S. lactis was essentially unchanged, but more than 10^7 E. coli were needed for a positive test. The ability of S. lactis to reduce resazurin in the absence of an electron carrier indicates that this organism has high Diaphorase activity, and the NADH-resazurin could detect 10^2 - 10^3 organisms. It is recommended that the ATP-glucose-resazurin reagents and NADH-resazurin be included when the CSL pathogen and background tests are repeated. One of these may be highly sensitive for some patholgens but insensitive to background. If used in combination with the INT test, this could permit discrimination between background and organisms not normally found in background samples.

Virus Test--Reaction conditions and apparatus necessary for conducting the virus test using nucleic acids and bacteriophage have been defined. However, these conditions are not suitable for CSL live virus

tests, where the virus is in a medium containing a relatively high concentration of fetal calf serum. Conditions suitable for the live virus test have not yet been identified, but the basic parameters of the test cannot be changed significantly. It is recommended that work toward resolving the difficulties in the live virus test be continued concurrently with the development of the equipment needed for field kits.

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