ABSTRACT

SUSAN W. ROGERS. A Quantitative Carrier Test for Disinfectant Testing Purposes. (Under the Direction of DR. LORI A TODD).

A quantitative carrier test for disinfectant testing was examined. Stainless steel penicylinders inoculated with bacteria were exposed to representative phenolic and quaternary ammonium compounds. Logarithmic reductions of the bacterial populations were obtained. The assay detected a dose-response relationship and gave reproducible results. The assay has the potential to replace the current sanctioned assay for disinfectant registration testing.

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Chapter 1

INTRODUCTION

There are hundreds of disinfectant formulations available for use in the home, industry or hospital setting, with a variety of intended uses that differ in both cost and performance. The Environmental Protection Agency (EPA) requires manufacturers of disinfectants to test their products for stability, human toxicity and effectiveness.[1] Prior to 1982, the EPA performed pre- and post-registration efficacy testing of disinfectants to verify the manufacturers' performance claims. Due to budget cutbacks this process was halted. [2] This has left a gap which state and hospital laboratories have been forced to fill.

While ineffective disinfectants marketed for home use may result in a monetary loss to the consumer, inadequate disinfectants used in a hospital setting can have both monetary and human health costs. Improperly disinfected patient care items can cause nosocomial infections when they contact sterile tissue, mucous membranes or non-intact skin. [3] Thus, the lack of testing, or the use of poorly designed tests of disinfectant performance, can lead to increased morbidity and the increased cost of lengthened hospital stays.

A clear, concise definition of the process of disinfection is necessary to understand the goals of disinfectant efficacy tests. Unfortunately, there are as many definitions of the term disinfection as there are disinfectants. In general, disinfection can be defined as a "process that eliminates all pathogenic microorganisms on inanimate objects with the exception of bacterial endospores." [4] From this definition, pathogenic microorganisms include vegetative bacteria, fungi, viruses, and protozoans capable of causing human disease. The exclusion of bacterial endospores, which are extremely hardy bacterial forms, sets limits on the process and differentiates it from sterilization in which all microorganisms are killed. The inclusion of the

term inanimate objects serves to differentiate the process from that of using antiseptics, which are applied to skin surfaces.

This research examined the laboratory tests available for disinfectant efficacy and determined those factors which constitute reproducible, efficient and cost effective tests. After reviewing current test methods, a new test method was developed and tested. This new method was designed to simplify and standardize disinfectant testing for laboratories that now individually certify new products. The data obtained with the new method provides insight into the performance of individual disinfectants.

Chapter 2

FACTORS INVOLVED IN DISINFECTANT TESTING

The purpose of all disinfectant tests is to assess the ability of a specific disinfectant to kill a bacterial load under prescribed conditions. The conditions of the test have an important impact on the ability of a disinfectant to perform as required. Significant parameters include the types of organisms to be killed, pH, contact time, presence of organic matter, temperature, disinfectant concentration and the presence of disinfectant neutralizers. To insure reproducibility, these conditions must be kept constant during the testing.

Choice of Organisms

Bacteria are the organisms chosen for disinfectant assays because they are easily grown and widely available. The specific bacteria chosen can have a significant impact on the outcome of the assay. Generally, more than one bacterial genus is used for a particular test, with at least one being a gram negative rod and one a gram positive cocci. There are two reasons for using multiple organisms: 1) in hospitals, contamination usually occurs with a variety of organisms, not a single species, and 2) the use of multiple organisms insures that the disinfectant was not formulated merely to kill a specific organism for a particular assay.[4]

In addition to choosing more than one organism for a particular test, it is necessary to choose the more resistant organisms, such as strains of Pseudomonas aeruginosa. It is generally thought that if a particular disinfectant can kill resistant organisms then it will also be able to kill less resistant organisms. [5]

The organisms chosen for a disinfectant test should be obtained from a culture collection such as the American Type Culture Collection (ATCC). This insures that all labs are using the same bacterial strain and that the organisms do not lose their intrinsic resistance from repeated subculture.[6]

pH

Hydrogen ion concentration, or pH, can be critical to disinfectant activity. Changes in the pH of a disinfectant solution can increase or decrease disinfectant effectiveness due to changes in the cell walls of the bacteria. Changes in pH can also alter the initial disinfectant structure. An increase in the pH has been found to result in decreased activity of phenols, chlorine compounds, organic acids and sulphur compounds, while a decrease in pH has been found to increase the activity of quaternary ammonium compounds. [7]

Close attention must be applied to the determination and maintenance of proper pH during testing, because a slight change can have a large effect on the outcome. The manufacturer's recommended pH guidelines should be observed not only during test situations but during in-use situations as well.

Contact time

Contact time between the bacterial load and disinfectant solution is also critical to effective killing. In most instances, increased contact time leads to increased effectiveness. The ideal contact time is a function of the disinfectant solution and the size of the bacterial load to be killed.[8]

Organic Matter

Surfaces to be disinfected generally contain organic matter such as blood and dirt. While the process of cleaning surfaces prior to disinfection is advocated, this suggestion is not always followed. In some situations, areas may not be accessible to cleaning. For this reason, some disinfectant tests challenge the ability of a disinfectant to overcome organic interference.

The presence of organic matter is thought to interfere with disinfectant activity by two mechanisms: 1) the presence of organic matter may shelter organisms from contact with the disinfectant and 2) when disinfectants bind with organic matter rather than organisms, the activity is decreased.[9]

The effect of organic matter on disinfectants depends on the composition of the organic matter and the disinfectant structure. A general rule is that the more chemically reactive the compound, the more it is affected by the presence of organic matter.[9] One study found that the higher the protein content of the organic matter, the higher the interference with disinfectants.[10] The same study concluded that doubling the disinfectant concentration did not significantly alter the neutralization by the organic matter.

Temperature

The temperature at which disinfectant tests are performed is another important consideration. The effect of temperature is related to disinfectant formulation and the bacterial cell growth kinetics.[11] Each disinfectant has its own critical temperature. One study found that glutaraldehyde was greatly affected by temperature while sodium hypochlorite was generally insensitive to temperature increases. This study also concluded that at temperatures above 50°C, any additional increase in killing was likely due to the heat sensitivity of the bacterial cells instead of increased disinfectant activity.[9]

The temperature at which the test organisms are grown and the temperature at which survivors are cultured is also important. There is some evidence that temperature changes can have an impact on the resistance patterns of some bacteria; those exposed to gamma radiation and chemical exposure may recover better at reduced temperatures.[5]

Disinfectant Concentration

Disinfectants are rarely received at their proper use-dilution for field use and laboratory tests; the user is responsible for adhering to the manufacturer's suggested dilution with the proper diluent. This is yet another source of error in field use and laboratory tests. According to Gray, the greatest continuing problem with disinfectants is the routine use of such products at ineffective concentrations.[12]

Some disinfectant assays test variations of the manufacturer's recommended dilutions to assess the margins of safety. The original Sykes test, for example, used two dilutions in addition to the manufacturer's recommended dilution.[13] Another study measured the efficacy of freshly prepared dilutions against the same dilutions that were several days old. Results demonstrated that proper dilutions could lose their effectiveness over time.[14]

Neutralizers

In all disinfectant assays it is necessary to have some method of neutralizing the disinfecting agent at the conclusion of the test. If neutralization is not accomplished, there will inevitably be some carryover into the recovery medium. This will extend the time of disinfection and can lead to erroneous results. To be effective, the neutralizer must inactivate the chosen disinfectant rapidly, without being inhibitory to the bacterial survivors. [15]

Some examples of commonly used neutralizers include Letheen media, which is capable of inactivating the quaternary compounds and Tween 80 which can neutralize the phenolics, hexachlorophene and formalin.[4] In addition, if a filter is used as a growth medium for surviving bacteria, it is necessary to wash the filter so that no residual disinfectant is left on the surface to inhibit growth.[16]

Chapter 3

CURRENT TESTING METHOD

The current, sanctioned method of testing disinfectants for registration purposes is the Association of Official Analytical Chemists' Use-Dilution Method (UDM). Problems associated with the current disinfection assay have led to the development of the Quantitative Carrier Test. The specific problems associated with the AOAC UDM are described below.

The AOAC's Use-Dilution Method is the sanctioned method in the United States for registering disinfectants with bactericidal activity. Manufacturers use the AOAC UDM to compile data for product registration with the Environmental Protection Agency. In the past, the EPA also used the UDM to verify the manufacturer's claims. This practice has been discontinued due to budget constraints.[2] Thus, new disinfectants are being permanently registered as efficacious on the basis of a test performed by the manufacturer which has been documented as having poor reproducibility.[32] Although the deficiencies of the UDM have been explored, no official attempts have been made to replace the assay with a more effective method. Attempts to improve the assay have not demonstrated the desired results.[32]

The AOAC UDM has a lengthy and complicated procedure. (See Appendix) It was developed in the 1950's and is a qualitative carrier test that uses phenol as a reference standard to measure the intrinsic resistance of the test organisms.

The major deficiency of the UDM is its' poor reproducibility. Studies have focused on the penicylinders, which act as carriers in the assay, and lack of good laboratory practice as potential sources of variability.[32] The assay is complex and requires attention to detail. Those laboratories using the UDM should perform the assay with some frequency in order to maintain a level of competency.

In an assay as long as the UDM there are many steps in which ambiguities can arise. The UDM Task Force sought to improve the test

reproducibility by recommending 32 changes to the approved method.

These changes were implemented in a collaborative study involving twelve laboratories. The study found that the interlaboratory variability was still high. This led the authors to concur with previous studies which had cited the penicylinders as a main source of variability.[32]

The AOAC method specifies the use of stainless steel penicylinders from S & L Metal Products Corporation. One study found under scanning electron microscopy that the penicylinders exhibited deep grooves and pitting. These defects are capable of protecting the bacteria from disinfectant contact. Glass penicylinders were examined and were found to be quite smooth; however there are difficulties in obtaining good bacterial adherence on such a surface. Porcelain penicylinders were also examined, as the EPA requires their use when the disinfectant is said to be effective on porous surfaces. The porcelain carriers had very rough surfaces and were not recommended for use in the UDM. [34,35]

A later study compared the S & L Metal Products penicylinder and that of Fisher Scientific. S & L Metal Products had halted production of the penicylinder for some time, forcing laboratories to use the Fisher brand. When examined microscopically, the Fisher brand had many of the same defects as the suggested brand. However, when both types were tested against a quaternary ammonium compound and a phenolic compound, the Fisher brand repeatedly had more failing tubes than the S & L Metal Products penicylinders. This is a source of inter- and intralaboratory variability. [36]

Another major source of variability in the AOAC UDM is the unequal challenge to the disinfectant caused by differences in bacterial attachment to the cylinders. Cole et al found significant differences in the mean numbers of bacteria attached to the cylinders: Pseudomonas aeruginosa had 1×10^7 organisms/cylinder, Staphylococcus aureus had 5×10^6 organisms/cylinder and Salmonella choleraesuis had 1×10^6 organisms/cylinder. A possible source of variability was thought to be the AOAC's use of unadjusted 48-54 hour broth suspensions. This study had a procedure modification which involved the use of McFarland turbidity standards to macroscopically adjust the number of bacterial cells present. McFarland standards are solutions of sulfuric acid and barium chloride, the densities of which approximate specific bacterial loads. A 1.0 McFarland approximates a bacterial density of 3.0×10^8 organisms/ml, while a 0.5 McFarland approximates 1.5×10^8 organisms/ml.

The study used Salmonella choleraesuis unadjusted, while the S. aureus broth was adjusted with phosphate buffered dilution water to a 1.0 McFarland and the P. aeruginosa broth was adjusted to match the turbidity of a 0.5 McFarland. The interlaboratory study using this modification still exhibited high levels of variability. However, it was thought that the variability was increased, in part, by the slight differences in overall methodology used by individual laboratories.[37]

Cole and Rutala conducted a second study, this time using 24 hour adjusted broth cultures instead of 48-54 hour cultures. The 18-24 hour cultures represent the peak of the cell growth curve. The Salmonella choleraesuis broth was not diluted and was found to have a mean attachment of 3.1×10^6 cells/penicylinder. This represented 0.38% of the original inoculum becoming attached to the cylinders. The Pseudomonas aeruginosa broth was adjusted to a 0.5 McFarland and had 3% attachment with a mean value of 3.0×10^7 cells/penicylinder. The Staphylococcus aureus broth was adjusted to a 1.0 McFarland and the number of organisms attaching was quite similar to that of S. choleraesuis. The attachment of P. aeruginosa, however, was statistically different from the other two organisms and further adjustments would be necessary to achieve comparable bacterial loads.[38]

The Pseudomonas aeruginosa broth can present special problems when inoculating penicylinders. Pseudomonads can form a pellicle at the top of the suspension and, if it is not properly removed, the result can be large fragments of organisms left in the tube. These pellets can be large enough to protect other organisms from disinfectant contact if they become attached to the penicylinder. It is important that the cell pellicle be removed by pipette suctioning followed by decanting in order to completely remove the pellicle.[42] This method, along with the use of the McFarland standard adjustment, still has not brought the penicylinder load of Pseudomonas aeruginosa in line with that of the other two organisms.

A second portion of the AOAC UDM results in additional differences in the number of cells attached to the penicylinders. When a carrier, such as a penicylinder, is immersed in a solution, organisms may be washed from the surface of the carrier. With this washoff, organisms will not be transferred into the recovery medium for possible detection. One study immersed bacteria-laden cylinders into a tube of saline for ten minutes. At the conclusion of the exposure, 40% of the carriers had 10% or less of the original

load still attached. No cylinder had over 50% of the original inoculum attached. Drying the cylinders for an extended time period had no impact on the strength of bacterial attachment to the cylinders.[39]

An additional study also attempted to quantitate the numbers of bacteria washed from the penicylinders during immersion. The cylinders were immersed in phosphate buffered dilution water for ten minutes. Approximately 89.9% of the S. choleraesuis, 48.8% of the P. aeruginosa and 38.8% of the S. aureus was washed off the cylinders. As the authors noted, the figures would have been much higher if the immersion had been in a disinfectant containing surfactants.[40]

The problem of initial bacterial attachment and bacterial washoff is significant and has not been addressed by the AOAC. Differences in numbers of bacterial cells on the cylinders results in unequal challenges to the disinfectant and killing time is directly related to the bacterial load.[40] With such significant differences in bacterial loads, it is not surprising that the AOAC UDM has a reproducibility problem.

While most problems with the AOAC UDM are attributed to procedures and equipment, some argue that it is the statistics of the assay that are at fault. Walter and Trout state that there are two important points that are overlooked when examining AOAC data: 1) a distribution of responses is always obtained when replicate testing is performed on the same sample and 2) if an acceptable response is prescribed, then there should also be an acceptable frequency of obtaining this response. The authors feel that the problem with the test is the lack of understanding concerning the expected variability.[41]

The AOAC method states that 59 of 60 cylinders must pass to maintain the 95% confidence limits.[33] Walter and Trout state that this is a vague statement and can actually lead to two interpretations of the statistics of the test. First, one failure in sixty cylinders could be seen as a probability of failure of 0.0167 (1/60), and this could be used as the upper boundary of the 95% confidence limit. If this interpretation is used, then the average probability of failure is much lower at 0.006. With the required three replicate tests, the average probability would fall to approximately 0.003. The second proposed interpretation is that 0.0167 is the average probability of failure and if small numbers of cylinders are tested then the confidence limits of the assay would be quite large. Thus, the authors argue that the variability of the AOAC UDM

is not totally due to poor test design but is in part due to a poor interpretation of the statistics of the assay.[41]

The study of the statistics of the AOAC UDM attempts to identify a standardized method of calculating a disinfectant concentration whose failure probability will be less than 0.0167 95% of the time. To do this, various disinfectant concentrations are tested and the results are plotted as probit versus log dose. From this data, the line of best fit is drawn and the 95% confidence boundaries are calculated. A dose on the upper 95% boundary would result in an expected result 95% of the time. This approach is thought to provide more accurate registration data while still retaining the original AOAC method.[41]

Another problem with the AOAC UDM is the use of phenol as the internal standard to assure that the test organisms are maintaining their resistance patterns. However, there is no procedure to insure that the person performing the test is correctly following the protocol since the UDM uses the phenol coefficient method instead of the actual UDM. This test differs widely from the UDM and it would be more valid to actually test phenol with the method. If a continuous response to phenol is assumed, then the results of the phenol UDM could be plotted on a Levy Jennings plot, which plots daily performance by mean and standard deviation. Mean and standard deviations are reviewed monthly to check for any shifts or trends, which would indicate that some portion of the assay was in error.

The AOAC UDM is a long and complicated assay that presents many opportunities for error during performance. The test must be performed by experienced technologists for acceptable degrees of reproducibility. The use of a qualitative carrier test, although sanctioned, does not guarantee that ineffective disinfectant concentrations will not be deemed as acceptable. When ineffective disinfectant concentrations are used in a hospital setting the result can be increased morbidity and mortality. The seriousness of this problem suggests that new disinfectant assays be examined in order to improve the registration process. A quantitative carrier test, for example, will present more accurate information on the killing ability of disinfectants.

Chapter 4

EXPERIMENTAL METHOD AND DESIGN

Experimental Procedure

The Materials of the QCT are located in the Appendix for comparison with those of the sanctioned AOAC Use-Dilution Method.

Pseudomonas aeruginosa, Salmonella choleraesuis and Staphylococcus aureus were tested a number of times with four registered disinfectants. Two phenolics, Tergisyl and Vesphene IIse, and two quaternary ammonium compounds, Buckeye and Tor, were tested for this study. Each disinfectant was diluted over a range of dilutions, including the recommended use-dilution. Exposure times for the study were one minute.

The 48-54 hour old organism suspension was pipetted into a small sterile flask containing a magnetic stir bar. The solution was mixed for approximately ten minutes to disperse any large clumps which would adhere to the penicylinders.

The penicylinders were drained of asparagine and removed from storage with a flamed wire hook to a sterile petri dish lined with filter paper. The penicylinders were placed upright and allowed to drain for approximately ten minutes.

The mixed organism suspension was pipetted into a 25 x 150 mm sterile test tube with a volume equal to the number of penicylinders to be tested. Using a flamed hook, the penicylinders were added to the suspension and allowed to sit undisturbed for 15 minutes. Using a flamed, cooled hook, the penicylinders were then removed from the suspension and placed upright on Whatman's No. 2 filter paper in a sterile petri dish. Penicylinders did not touch other penicylinders or the sides of the petri dish. The covered petri dish was placed in a 35^0C incubator for at least 20 minutes but not more than 60 minutes.

After the disinfectant tubes had reached 20^0C in a circulating waterbath, a penicylinder was placed into the dilution tube using a flamed, cooled hook.

The tube was gently swirled and the penicylinder was allowed to remain in the disinfectant for one minute. The temperature of the waterbath was constantly monitored using a Fisher LED thermometer placed in a test tube containing ten milliliters of sterile water. The penicylinder was transferred to a 20 ml tube of Letheen broth at the conclusion of the exposure period, with care being taken to drain off excess disinfectant. After all of the penicylinders had been placed in Letheen broth they were then individually transferred to a 10 ml tube of Tween 80 Saline.

Two randomly chosen penicylinders, not previously exposed to a disinfectant, were placed into individual Tween 80 Saline tubes and treated exactly as other penicylinders in order to quantitate the initial bacterial load present.

The Tween 80 Saline tubes containing the penicylinders were placed in the ultrasonic waterbath for ten minutes. They were then vortexed at setting number five for a period of two minutes.

For the quantitation penicylinders, 1 ml of the Tween 80 Saline was removed from the vortexed tube and placed in a 99 ml bottle of Tween 80 Saline and mixed. This resulted in a 1×10^3 dilution. One milliliter of this dilution was transferred to 99 mls of Tween 80 Saline to make a 1×10^5 dilution. Both dilutions were filtered using a hydrophobic grid membrane and counted to determine the initial bacterial numbers on the penicylinders.

The filter apparatus was assembled and for each penicylinder the corresponding Letheen broth and Tween 80 Saline tubes were filtered. Penicylinders were removed prior to filtering. The hydrophobic grid membrane was then rinsed with an additional 20 ml of Letheen broth to neutralize any disinfectant carryover. Upon completion of the filtering each grid was aseptically removed and placed grid side up on a Letheen agar plate. Plates were incubated at 35^0C for 48 hours.

Pour plates of the original inoculating broth were prepared by serially diluting the broth to 10^{-4}, 10^{-6},10^{-8}, and 10^{-10} in Trypticase Soy Broth to achieve countable numbers. One milliliter of each dilution was placed in 19 milliliters of warm Trypticase Soy Agar and poured into a sterile petri dish. Following 48 hours of incubation, the plates containing between 30 and 300 colonies were counted. From these counts, it was possible to check for purity and if desired, determine the percentage of organisms attaching to the penicylinders.

Each membrane grid contained 1600 squares (40 rows x 40 rows). If less than 400 colonies were present, all colonies were counted. If over 400 colonies were present, five random rows were counted, averaged and multiplied by 40 to obtain a count.

Following conclusion of the assay the filter assemblies were placed in an appropriate disinfectant solution for at least 10 minutes and then rinsed with copious amounts of water. Filters were then steam sterilized at 121^{0}C for 20 minutes prior to re-use.

For calculations the bacterial counts of the two quantitation penicylinders were averaged to determine the count per penicylinder. To determine the log reduction of each replicate the logarithm of the grid count was subtracted from the logarithm of the averaged count per penicylinder.

Chapter 5

EXPERIMENTAL RESULTS

One method of assessing the killing ability of a disinfectant involved comparing the log reduction of each replicate of a particular dilution with each replicate of the adjacent, higher dilution. There are three possible outcomes when the replicates are compared: 1) the log reduction of the lower dilution is higher than the log reduction of the next higher dilution, 2) the log reduction of the higher dilution is greater than that of the adjacent lower dilution, or 3) the log reductions of the two dilutions are equal or it is not possible to determine which reduction is greater (>5.6 logs vs >7.0 logs for example). The first outcome is a correct result, the second is an incorrect, or unexpected, outcome and the third outcome is an indeterminate result. This method examines the disinfectant performance over all dilutions tested, rather than only examining the use-dilution. Thus, some level of failure is to be expected. Results for each disinfectant class are listed in Tables 13 and 14.

Tergisyl, a phenolic was tested with each of the three bacterial strains. The results of Tergisyl with Pseudomonas aeruginosa are found in Table 1. Dilutions of 1:100, 1:200, 1:400 and 1:800 were tested, with 1:100 being the recommended use-dilution. The range of log reductions for the 1:100 dilution was 5.4 to >7.6 logs. For the highest dilution the log reductions decreased to an average of 4.3. Figure 1 depicts the decreasing log reductions with increasing disinfectant dilutions. (For graphing purposes, all greater than signs were dropped when computing average log reductions). Using the method described previously for comparing all replicates, the correct result was obtained 56% of the time. Thirteen percent of the responses were incorrect and 31% of the responses were indeterminate.

Salmonella choleraesuis was tested against the previous Tergisyl dilutions, with the addition of a 1:1600 dilution. The results are in Table 2 and Figure 2. The range of log reductions over all dilutions was 5.7 to 2.5 logs. The use-dilution, 1:100, had a range of 3.5 to >5.3 logs. The highest dilution,

1:1600, had a range of 2.5 to 2.9 logs. Forty-five percent of all responses were correct and 7% of the responses were incorrect. Forty-eight percent of the responses were indeterminate.

Staphylococcus aureus demonstrated a dose-response to Tergisyl. (Table 3, Figure 3). At the use-dilution of 1:100 the log reduction was >7.7 logs. The log reductions declined steadily until the 1:400 dilution. The log reduction ranged from 4.5 to 5.0 through the 1:1600 dilution. Fifty-three percent of all Staphylococcus responses were correct, 22% were incorrect and 25% of the responses were indeterminate.

The second phenolic, Vesphene IIse, was tested against Pseudomonas aeruginosa at dilutions of 1:128, 1:256 and 1:512. (Table 4). There is a dose-response over the three dilutions. (Figure 4). The use-dilution, 1:128, had a range of log reductions of >5.2 to >7.6 logs. At the highest dilution the range decreased to 2.0 to 4.3 logs. Examining all dilutions, 79% of the responses were correct and four percent were incorrect. Seventeen percent of all replicates were indeterminate.

Against Salmonella choleraesuis six doubling Vesphene IIse dilutions were tested, ending at 1:2048. A dose-response curve was obtained. (Figure 5). Log reductions ranged from 2.4 to >5.7 logs. (Table 5). Fifty-three percent of all replicates gave the correct, or expected result. Seven percent were incorrect and the remaining 37% were indeterminate.

Staphylococcus aureus was tested against the same six dilutions of Vesphene IIse and demonstrated the best response of the three organisms. A greater than 7.7 log reduction was obtained at the use-dilution. The lowest reduction obtained was 4.6 logs. (Table 6). The dose-response curve can be seen in Figure 6. Sixty-four percent of the responses were correct and 21% were incorrect.

A second class of disinfectants, quaternary ammonium compounds, was also tested against the three organisms. The first disinfectant of the class was Buckeye, which had a use-dilution of 1:256. Against Pseudomonas aeruginosa, doubling dilutions ranged from 1:128 to 1:2048. (Table 7). With one minute exposures a dose-response curve was not obtained. (Figure 7). The averages of the log reductions for the dilutions had a range of 5.1 to 5.7 logs. Comparing the replicates of adjacent dilutions, 46% were correct. Forty-eight percent of the responses were incorrect. The remaining six percent of the responses were indeterminate.

When Buckeye was challenged with Salmonella choleraesuis the log reductions ranged from 4.5 to >6.9 logs over dilutions spanning from 1:128 to 1:2048. (Table 8). A dose-response was not achieved. (Figure 8). The lowest dilution , 1:128 and the highest dilution, 1:2048, had identical average log reductions of 6.4. Fifteen percent of the responses were incorrect, 20% were correct and 65% of the responses were indeterminate.

Staphylococcus aureus was tested against Buckeye over the same five dilutions. Over five dilutions the range of log reductions was 4.8 to >7.8. (Table 9). A slight dose-response was observed. (Figure 9). For this organism an additional dilution of 1:16384 was tested. At this dilutions, 128 times more dilute than the use-dilution, the average log reduction was 4.2. The correct response was obtained in 65% of the runs and the incorrect response was obtained with 27% of the replicates.

Tor, the second quaternary ammonium disinfectant had a use-dilution of 1:64. With Pseudomonas aeruginosa the doubling dilutions ranged from 1:32 to 1:512. A slight dose-response was observed. (Figure 10). The range of log reductions was 4.4 to 8.2 logs. The 1:32 dilution, twice the strength of the use-dilution, had an average reduction of 6.4 logs. The use-dilution, 1:64, had an average reduction of 5.6 logs. Fifty-four percent of the responses were correct and 36% of the replicates gave incorrect responses.

The same dilutions of Tor were tested with Salmonella choleraesuis. A dose-response was achieved if the response of the 1:128 dilution is considered to be an outlier. (Figure 11). The overall log reduction range was 3.7 to >6.9 logs. Over all dilutions the correct response was obtained 40% of the time and the incorrect response was obtained for 32% of the replicates. Twenty eight percent of the replicates were indeterminate.

Against Staphylococcus aureus, Tor achieved the highest log reductions. (Table 12). The range of reductions was 5.2 to >7.8 logs. A good dose-response was not obtained. (Figure 12). The average log reduction for the use-dilution was 6.8 logs. Over all dilution replicates 45% were correct and 40% were indeterminate. Fifteen percent of the responses were indeterminate.

Table 1: Pseudomonas vs Tergisyl

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
7-9	Tergisyl	1:100	1.76×10^5	>5.2,>5.2,>5.2
7-23	Tergisyl	1:100	3.12×10^7	>7.5,>7.5
7-31	Tergisyl	1:100	3.57×10^7	>7.6,5.4,5.4
7-9	Tergisyl	1:200	1.76×10^5	>5.2,>5.2,3.4
7-23	Tergisyl	1:200	3.12×10^7	7.5,>7.5
7-31	Tergisyl	1:200	3.57×10^7	5.3,>7.6,>7.6
7-9	Tergisyl	1:400	1.76×10^5	2.0,2.1,2.3
7-23	Tergisyl	1:400	3.12×10^7	4.7,4.3,4.3
7-31	Tergisyl	1:400	3.57×10^7	4.3,4.4,4.3
7-23	Tergisyl	1:800	3.12×10^7	4.3,4.3

Figure 1: Pseudomonas vs Tergisyl

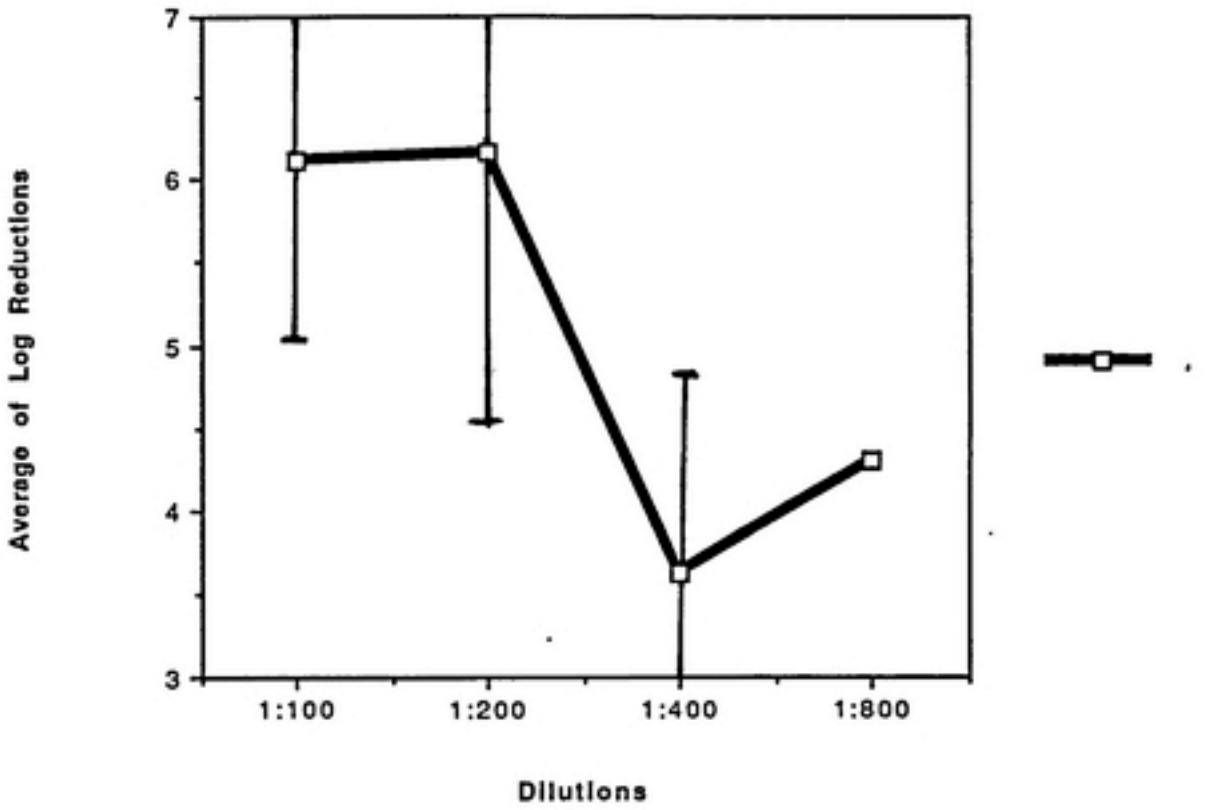

Table 2: Salmonella vs. Tergisyl

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
8-7	Tergisyl	1:100	2.15×10^5	>5.3,>5.3,>5.3
9-11	Tergisyl	1:100	4.7×10^4	>4.7,>4.7,3.5
8-7	Tergisyl	1:200	2.15×10^5	>5.3,>5.3,>5.3
9-11	Tergisyl	1:200	4.7×10^4	>4.7,>4.7,>4.7
8-7	Tergisyl	1:400	2.15×10^5	>5.3,>5.3,>5.3
9-11	Tergisyl	1:400	4.7×10^4	4.4,>4.7,>4.7
8-14	Tergisyl	1:400	1.0×10^5	3.8,4.4,4.7
9-10	Tergisyl	1:400	5.48×10^5	5.4,5.4,5.7
8-14	Tergisyl	1:800	1.07×10^5	2.6,2.8
9-10	Tergisyl	1:800	5.48×10^5	2.5,2.5
8-14	Tergisyl	1:1600	1.07×10^5	2.6,2.9
9-10	Tergisyl	1:1600	5.48×10^5	2.5,2.5,2.5

Figure 2: Salmonella vs Tergisyl

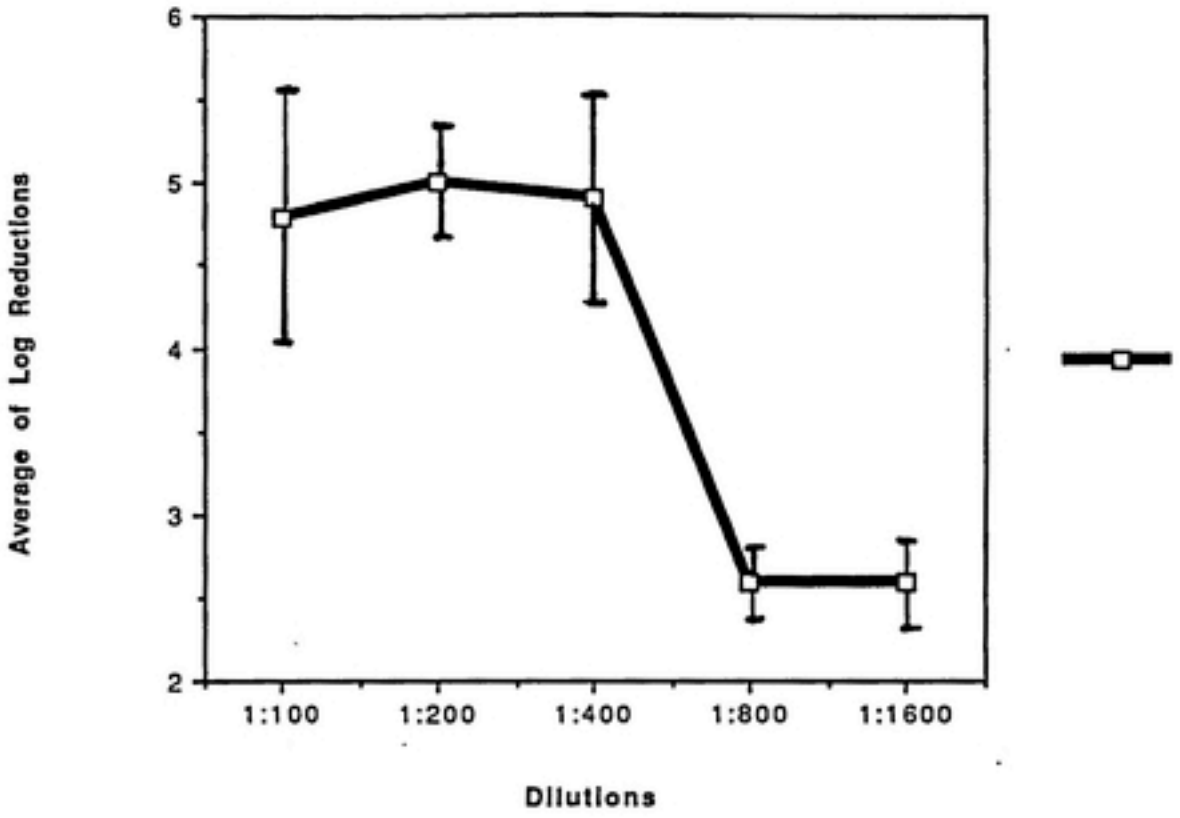

Table 3: S. aureus vs Tergisyl

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
9-9	Tergisyl	1:100	5.44×10^7	7.7,>7.7,7.7
9-12	Tergisyl	1:100	4.88×10^7	>7.7,>7.7,>7.7
9-9	Tergisyl	1:200	5.44×10^7	>7.7,7.4,>7.7
9-12	Tergisyl	1:200	4.88×10^7	7.4,7.4,>7.7
9-9	Tergisyl	1:400	5.44×10^7	4.5,4.5,4.5
9-12	Tergisyl	1:400	4.88×10^7	5.0,4.6,4.6
9-13	Tergisyl	1:400	6.12×10^7	4.6,4.6,4.6
9-16	Tergisyl	1:400	7.8×10^7	4.7,4.7,4.7
9-13	Tergisyl	1:800	6.12×10^7	4.6,4.6,4.6
9-16	Tergisyl	1:800	7.8×10^7	4.7,4,7,4,7
9-13	Tergisyl	1:1600	6.12×10^7	4.6,4.6,4.6
9-16	Tergisyl	1:1600	7.8×10^7	4.7,4.7,4.7

Figure 3: S. aureus vs Tergisyl

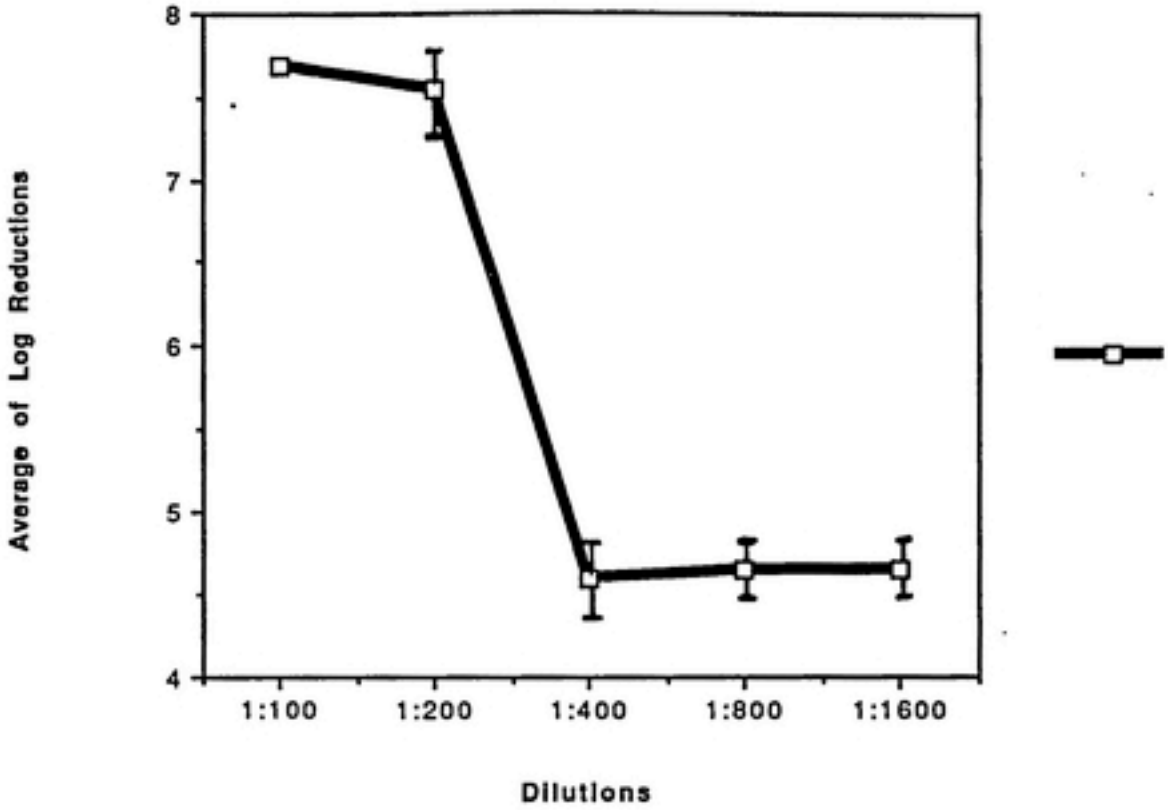

Table 4: Pseudomonas vs Vesphene IIse

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
7-9	Vesphene	1:128	1.76×10^5	>5.2,>5.2,>5.2
7-31	Vesphene	1:128	3.57×10^7	>7.6,>7.6,>7.6
7-9	Vesphene	1:256	1.76×10^5	2.0,4.5,>5.2
7-31	Vesphene	1:256	3.57×10^7	4.3,4.4,4.7
7-9	Vesphene	1:512	1.76×10^5	2.0,2.0
7-31	Vesphene	1:512	3.57×10^7	4.3,4.3,4.3

Figure 4: Pseudomonas vs Vesphene IIse

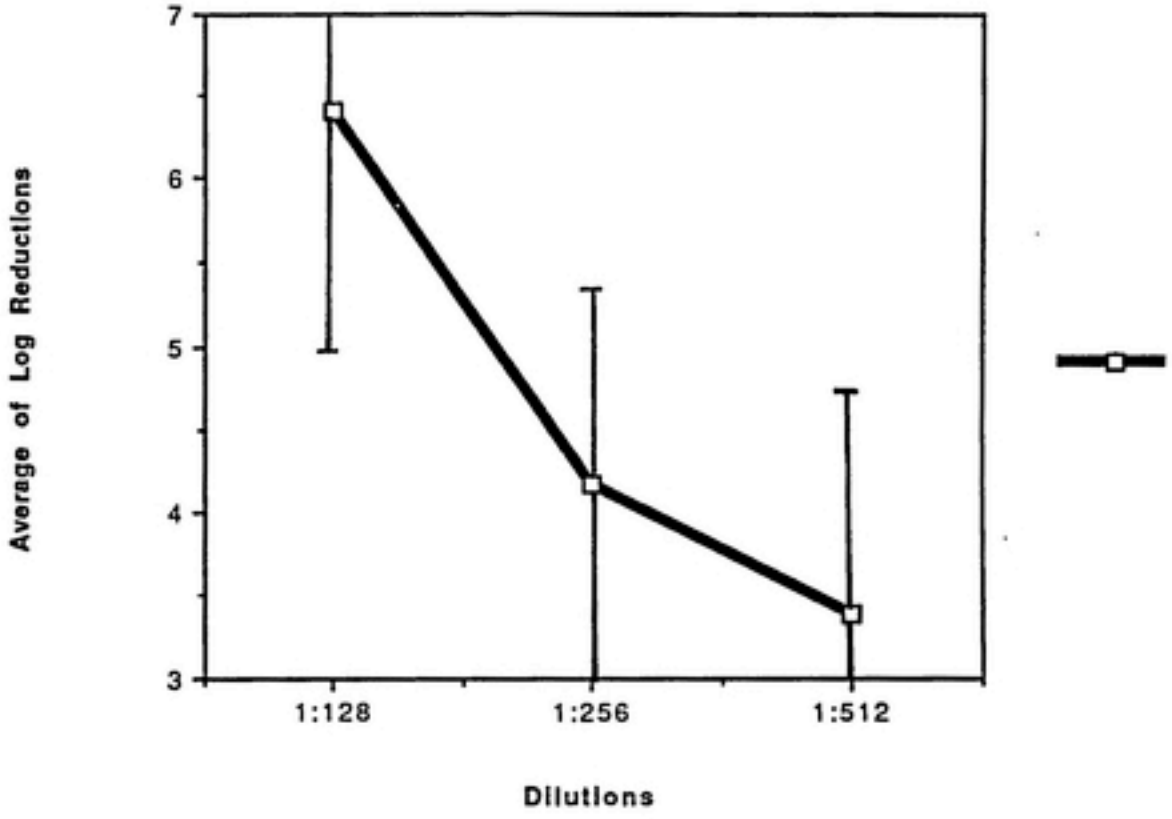

Table 5: Salmonella vs Vesphene IIse

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
8-7	Vesphene	1:128	2.15×10^5	>5.3,>5.3,5.3
9-11	Vesphene	1:128	4.7×10^4	>4.7,>4.7
8-7	Vesphene	1:256	2.15×10^5	>5.3,>5.3,>5.3
9-11	Vesphene	1:256	4.7×10^4	>4.7,>4.7
8-7	Vesphene	1:512	2.15×10^5	>5.3,5.3,5.0
8-14	Vesphene	1:512	1.07×10^5	>5.0.>5.0,5.0
9-10	Vesphene	1:512	5.48×10^5	>5.7,3.4,>5.7
9-11	Vesphene	1:512	4.7×10^4	>4.7,4.7,3.2
8-14	Vesphene	1:1024	1.07×10^5	3.0,2.8,2.7
9-10	Vesphene	1:1024	5.48×10^5	3.5,3.6
8-14	Vesphene	1:2048	1.07×10^5	2.7,3.1,2.4
9-10	Vesphene	1:2048	5.48×10^5	3.0,3.0

Figure 5: Salmonella vs Vesphene IIse

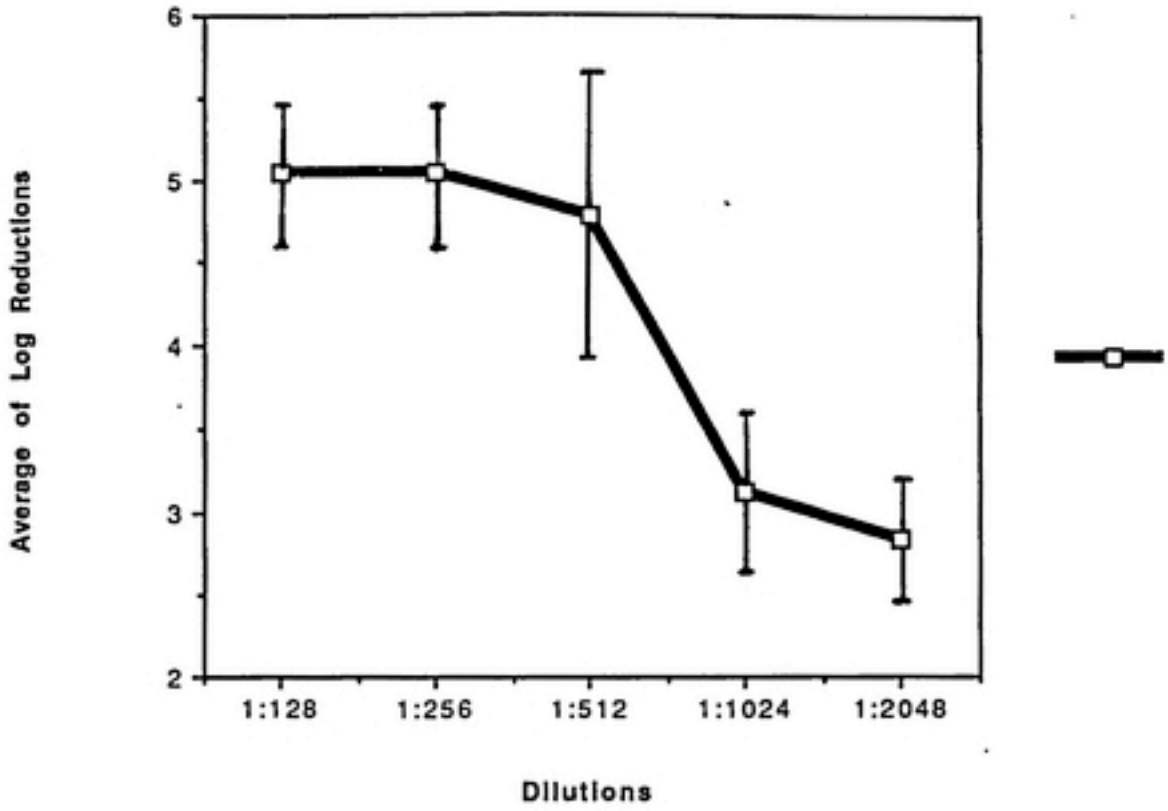

Table 6: S. aureus vs Vesphene IIse

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
9-9	Vesphene	1:128	5.44×10^7	7.7,>7.7,7.7
9-12	Vesphene	1:128	4.88×10^7	>7.7,>7.7,>7.7
9-9	Vesphene	1:256	5.44×10^7	5.5,4.9,5.1
9-12	Vesphene	1:256	4.88×10^7	5.8,6.3,6.0
9-9	Vesphene	1:512	5.44×10^7	4.5,4.5,4.5
9-12	Vesphene	1:512	4.88×10^7	4.5,4.5
9-13	Vesphene	1:512	6.12×10^7	4.6,4.6,4.6
9-16	Vesphene	1:512	7.8×10^7	4.7,4.7,4.7
9-13	Vesphene	1:1024	6.12×10^7	4.6,4.6
9-16	Vesphene	1:1024	7.8×10^7	4.7,4.7,4.7
9-13	Vesphene	1:2048	6.12×10^7	4.6,4.6,4.6
9-16	Vesphene	1:2048	7.8×10^7	4.7,4.7

Figure 6: S. aureus vs Vesphene IIse

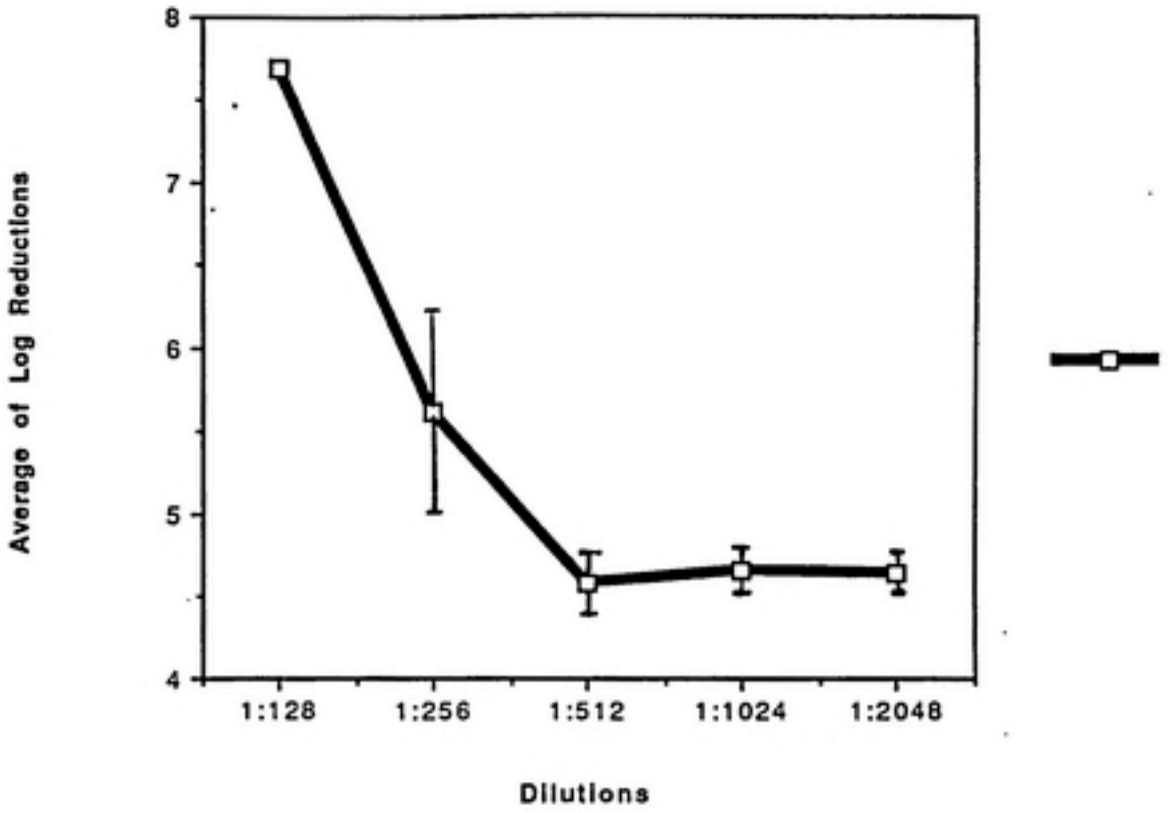

Table 7: Pseudomonas vs Buckeye

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
10-2	Buckeye	1:128	1.45×10^8	5.0,5.0
10-4	Buckeye	1:128	7.12×10^7	5.8,4.8
9-18	Buckeye	1:256	4.26×10^7	4.5,4.4,4.4
9-17	Buckeye	1:256	1.33×10^8	4.9,6.2,7.3
10-2	Buckeye	1:256	1.45×10^8	>8.2,5.0
10-4	Buckeye	1:256	7.12×10^7	5.7,4.7
9-18	Buckeye	1:512	4.26×10^7	6.3,4.4,6.6
9-17	Buckeye	1:512	1.33×10^8	5.0,5.7
10-2	Buckeye	1:512	1.45×10^8	5.0
10-4	Buckeye	1:512	7.12×10^7	6.6
9-18	Buckeye	1:1024	4.26×10^7	4.8,4.9
9-17	Buckeye	1:1024	1.33×10^8	4.9,4.9,5.2
10-2	Buckeye	1:1024	1.45×10^8	5.0,5.2
10-4	Buckeye	1:1024	7.12×10^7	6.3,4.6
10-2	Buckeye	1:2048	1.45×10^8	5.0
10-4	Buckeye	1:2048	7.12×10^7	5.6

Figure 7: Pseudomonas vs Buckeye

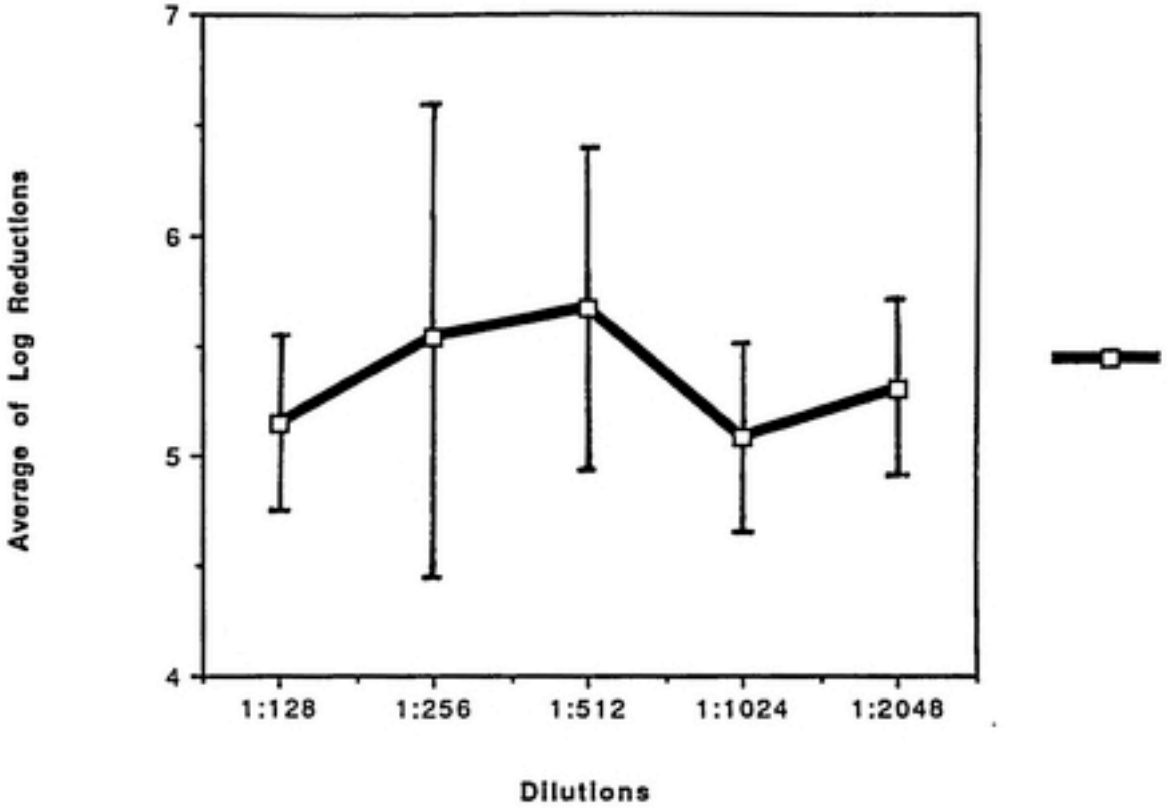

Table 8: Salmonella vs Buckeye

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
10-8	Buckeye	1:128	9.96×10^5	>6.0,>6.0
10-9	Buckeye	1:128	7.3×10^6	>6.9,>6.9
10-8	Buckeye	1:256	9.96×10^5	>6.0,>6.0
10-9	Buckeye	1:256	7.3×10^6	>6.9,4.5
10-8	Buckeye	1:512	9.96×10^5	>6.0
10-9	Buckeye	1:512	7.3×10^6	>6.9
10-8	Buckeye	1:1024	9.96×10^5	>6.0,>6.0
10-9	Buckeye	1:1024	7.3×10^6	6.3,5.9
10-8	Buckeye	1:2048	9.96×10^5	>6.0
10-9	Buckeye	1:2048	7.3×10^6	>6.9

Figure 8: Salmonella vs Buckeye

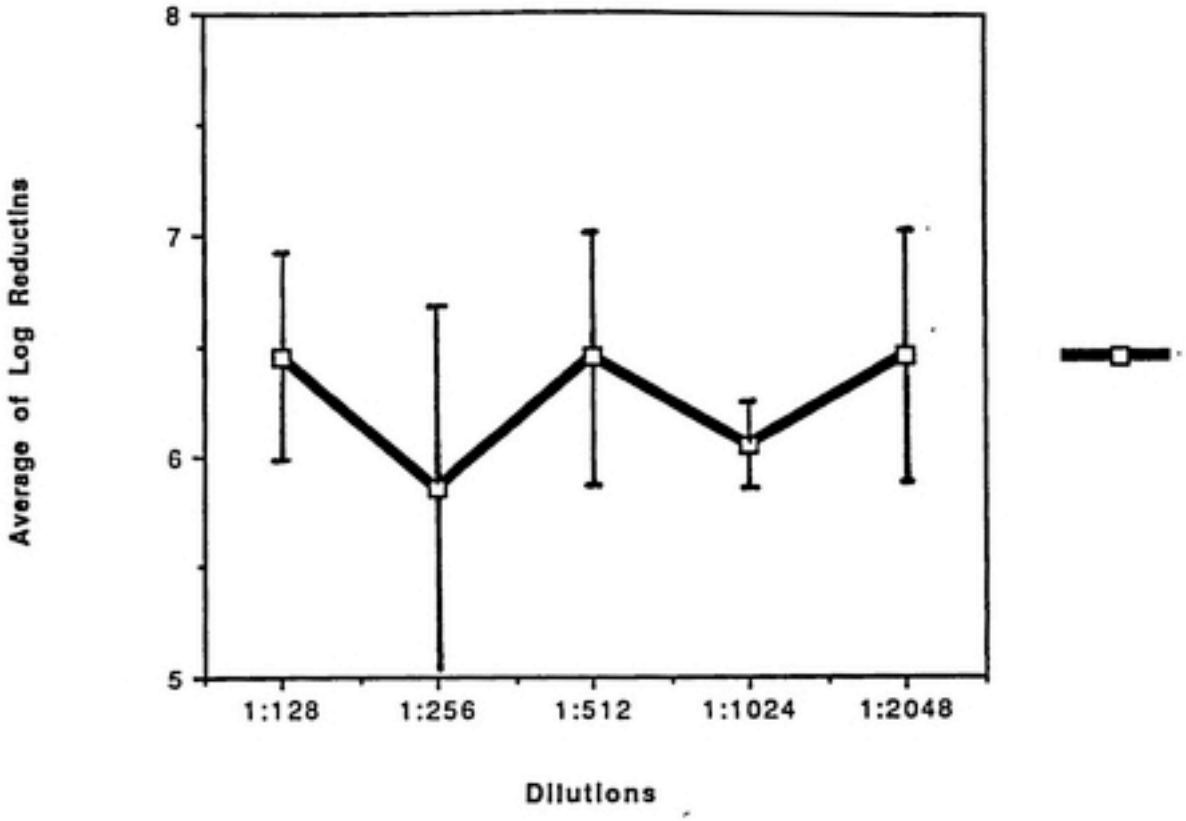

Table 9: S. aureus vs Buckeye

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
9-27	Buckeye	1:128	7.24×10^7	6.2,>7.8
10-1	Buckeye	1:128	3.35×10^7	>7.5,6.6
9-27	Buckeye	1:256	7.24×10^7	6.4,>7.8
10-1	Buckeye	1:256	3.35×10^7	>7.5,6.4
9-27	Buckeye	1:512	7.24×10^7	5.0
10-1	Buckeye	1:512	3.35×10^7	7.5
9-27	Buckeye	1:1024	7.24×10^7	5.0,7.2
10-1	Buckeye	1:1024	3.35×10^7	>7.5,>7.5
9-27	Buckeye	1:2048	7.24×10^7	5.9
10-1	Buckeye	1:2048	3.35×10^7	4.8

Figure 9: S. aureus vs Buckeye

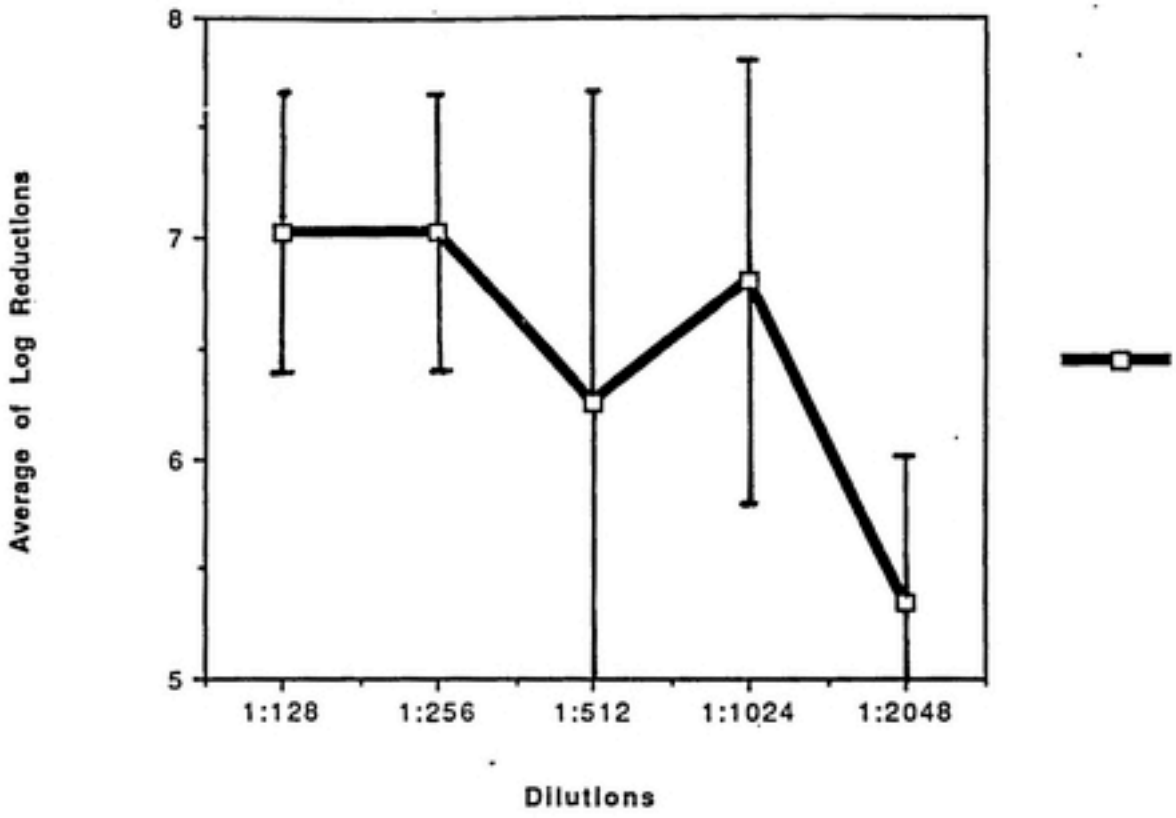

Table 10: Pseudomonas vs Tor

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
10-2	Tor	1:32	1.45×10^8	8.2,5.0
10-4	Tor	1:32	7.12×10^7	7.8,4.6
9-18	Tor	1:64	4.26×10^7	4.7,5.3,7.3
9-17	Tor	1:64	1.33×10^8	4.9,5.1,5.0
10-2	Tor	1:64	1.45×10^8	5.0,5.0
10-5	Tor	1:64	7.12×10^7	>7.8
9-18	Tor	1:128	4.26×10^7	4.4,4.4,4.6
9-17	Tor	1:128	1.33×10^8	4.9,5,8
10-2	Tor	1:128	1.45×10^8	5.0
10-4	Tor	1:128	7.12×10^7	7.2
9-18	Tor	1:256	4.26×10^7	4.4,4.4
9-17	Tor	1:256	1.33×10^8	4.9,4.9,4.9
10-2	Tor	1:256	1.45×10^8	5.1,5.0
10-4	Tor	1:256	7.12×10^7	4.6,4.6
10-2	Tor	1:512	1.45×10^8	5.0
10-4	Tor	1:512	7.12×10^7	4.8

Figure 10: Pseudomonas vs Tor

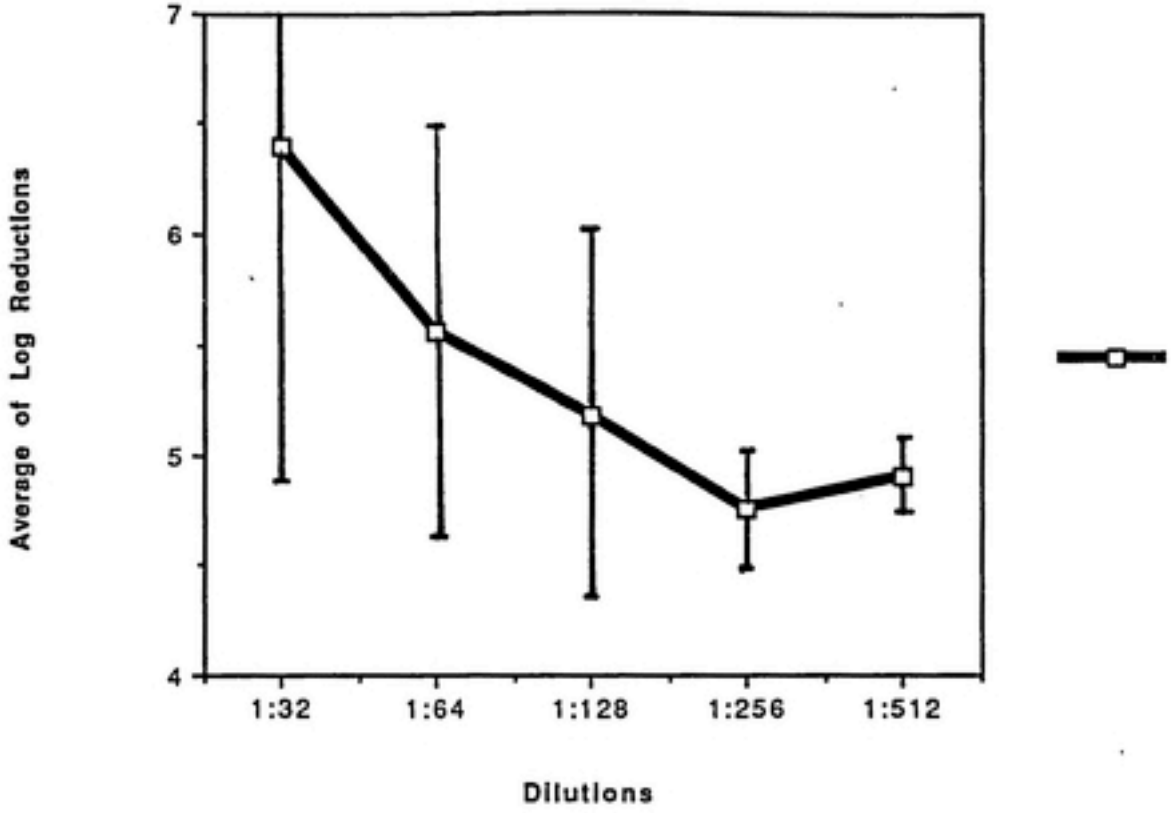

Table 11: Salmonella vs Tor

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
10-8	Tor	1:32	9.96×10^5	>6.0,6.0
10-9	Tor	1:32	7.3×10^6	>6.9,5.3
10-8	Tor	1:64	9.96×10^5	5.5,>6.0
10-9	Tor	1:64	7.3×10^6	4.5,>6.9
10-8	Tor	1:128	9.96×10^5	>6.0
10-9	Tor	1:128	7.3×10^6	6.9
10-8	Tor	1:256	9.96×10^5	>6.0,>6.0
10-9	Tor	1:256	7.3×10^6	4.0,5.1
10-8	Tor	1:512	9.96×10^5	3.7
10-9	Tor	1:512	7.3×10^6	6.1

Figure 11: Salmonella vs Tor

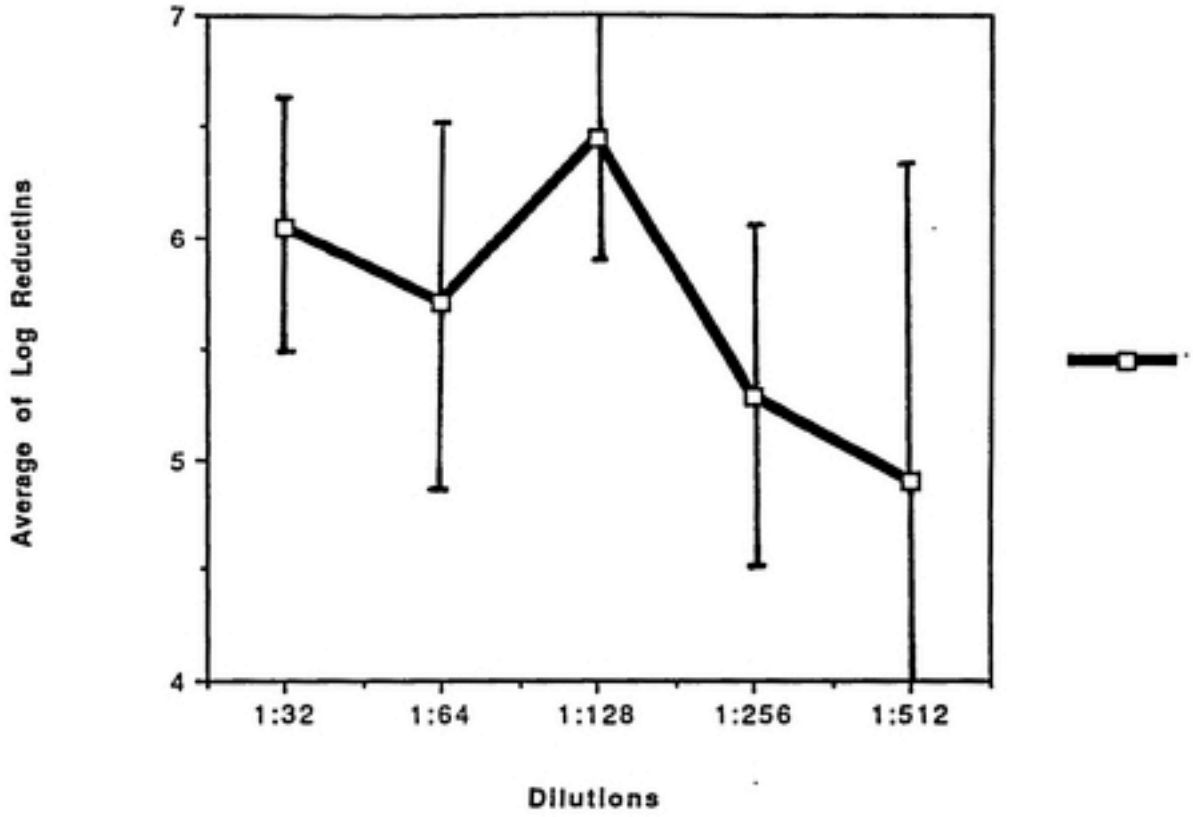

Table 12: S.aureus vs Tor

Date	Disinfectant	Dilution	Orgs/Penicylinder	Log Reduction
9-27	Tor	1:32	7.24×10^7	>7.8,6.0
10-1	Tor	1:32	3.35×10^7	>7.5,>7.5
9-27	Tor	1:64	7.24×10^7	6.2,6.2
10-1	Tor	1:64	3.35×10^7	>7.5,>7.5
9-27	Tor	1:128	7.24×10^7	6.4
10-1	Tor	1:128	3.35×10^7	6.0
9-27	Tor	1:256	7.24×10^7	7.6,7.4
10-1	Tor	1:256	3.35×10^7	5.8,7.5
9-27	Tor	1:512	7.24×10^7	7.8
10-1	Tor	1:512	3.35×10^7	5.2

Figure 12: S. aureus vs Tor

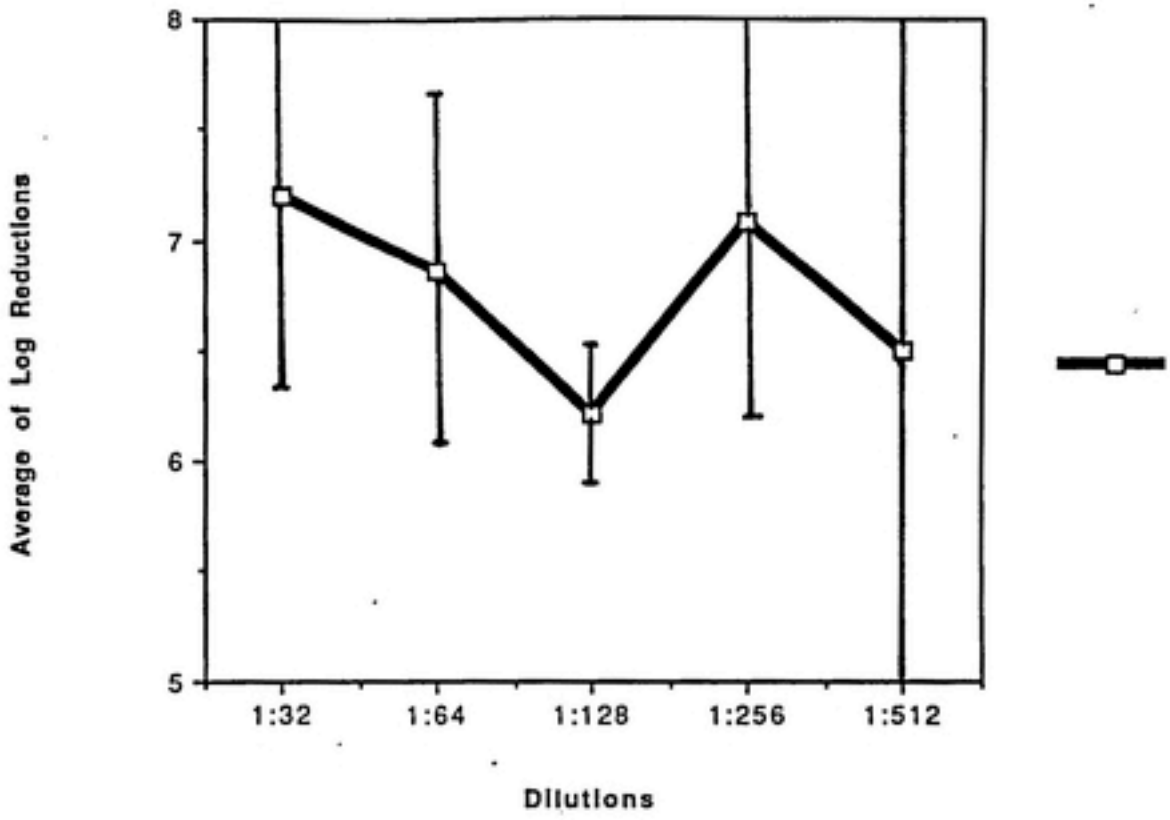

TABLE 13: DOSE RESPONSE OF PHENOLICS

COMBINATION	DILUTIONS	CORRECT[a]	INCORRECT[b]	INDETERMINATE
Pseudo/Tergisyl	1:100 v 1:200 (n=64)	16 (25%)	8 (12.5%)	40 (62.5%)
	1:200 v 1:400 (n=72)	66 (92%)	6 (8%)	0
	1:400 v 1:800 (n=18)	4 (22.2%)	6 (33.3%)	8(44.4%)
Overall%	Total=154	86(56%)	20(13%)	48(31%)
Salmonella/Tergisyl	1:100 v 1:200 (n=36)	0	6 (17%)	30 (83%)
	1:200 v 1:400 (n=72)	24 (33%)	0	48 (67%)
	1:400 v 1:800 (n=48)	48 (100%)	0	0
	1:800 v 1:1600 (n=20)	7 (35%)	6(30%)	7(35%)
Overall%	Total= 176	79 (45%)	12(7%)	85(48%)
Staph/Tergisyl	1:100 v 1:200 (n=36)	18 (50%)	6 (17%)	12 (33%)
	1:200 v 1:400 (n=72)	72 (100%)	0	0
	1:400 v 1:800 (n=72)	15 (21%)	33 (46%)	24 (33%)
	1:800 v 1:1600 (n=36)	9 (25%)	9 (25%)	18 (50%)
Overall %	Total=216	114 (53%)	48 (22%)	54 (25%)
Pseudo/Vesphene	1:128 v 1:256 (n=36)	30 (83%)	0	6 (17%)
	1:256 v 1:512 (n=30)	22 (73%)	3 (10%)	5 (17%)
Overall %	Total= 66	52 (79%)	3 (4%)	11 (17%)
Salmonella/Vesphene	1:128 v 1:256 (n=25)	0	0	25 (100%)
	1:256 v 1:512 (n=60)	24 (40%)	0	36 (60%)
	1:512 v 1:1024 (n=60)	56 (93%)	4 (7%)	0
	1:1024 v 1:2048 (n=25)	15 (60%)	8 (32%)	2 (8%)
Overall %	Total= 170	95 (56%)	12 (7%)	63 (37%)
Staph/Vesphene	1:128 v 1:256 (n=36)	36 (100%)	0	0
	1:256 v 1:512 (n=66)	66 (100%)	0	0
	1:512 v 1:1024 (n=55)	6 (11%)	34 (62%)	15 (27%)
	1:1024 v 1:2048 (n=25)	9 (36%)	4 (16%)	12 (48%)
Overall %	Total= 182	117 (64%)	38 (21%)	27 (15%)

[a] : Lower dilution with higher kill [b]: Higher dilution with higher kill

TABLE 14: DOSE RESPONSE OF QUATERNARY AMMONIUM COMPOUNDS

COMBINATION	DILUTION	CORRECT[a]	INCORRECT[b]	INDETERMINATE
Pseudo/Buckeye	1:128 v 1:256 (n=40)	21 (52%)	17 (43%)	2 (5%)
	1:256 v 1:512 (n=70)	19 (27%)	46 (66%)	5 (7%)
	1:512 v 1:1024 (n= 63)	43 (68%)	16 (25%)	4 (6%)
	1:1024 v 1:2048 (n=18)	4 (22%)	13 (72%)	1 (6%)
Overall %	Total= 191	87 (46%)	92 (48%)	12 (6%)
Salmonella/Buckeye	1:128 v 1:256 (n=16)	4 (25%)	0	12 (75%)
	1:256 v 1:512 (n=8)	0	2 (25%)	6 (75%)
	1:512 v 1:1024 (n=8)	4 (50%)	0	4(50%)
	1:1024 v 1:2048 (n=8)	0	4 (50%)	4 (50%)
Overall %	Total= 40	8 (20%)	6 (15%)	26 (65%)
Staph/Buckeye	1:128 v 1:256 (n=28)	12 (43%)	10 (36%)	6 (21%)
	1:256 v 1:512 (n=14)	10 (71%)	4 (29%)	0
	1:512 v 1:1024 (n=8)	2 (25%)	5 (63%)	1 (12%)
	1:1024 v 1:2048 (n=20)	16 (80%)	4 (20%)	0
	1:2048 v 1:16384 (n=15)	15 (100%)	0	0
Overall %	Total= 85	55 (65%)	23 (27%)	7 (8%)
Pseudo/Tor	1:32 v 1:64 (n=36)	18 (50%)	14 (39%)	4 (11%)
	1:64 v 1:128 (n=63)	42 (67%)	17 (27%)	4 (6%)
	1:128 v 1:256 (n=63)	31 (49%)	22 (35%)	10 (16%)
	1:256 v 1:512 (n=18)	6 (33%)	11 (61%)	1 (6%)
Overall %	Total= 180	97 (54%)	64 (36%)	19 (10%)
Salmonella/Tor	1:32 v 1:64 (n=16)	7 (44%)	5 (31%)	4 (25%)
	1:64 v 1:128 (n=8)	1 (12%)	4 (50%)	3 (38%)
	1:128 v 1:256 (n=8)	4 (50%)	2 (25%)	2 (25%)
	1:256 v 1:512 (n=8)	4 (50%)	2 (25%)	2 (25%)
Overall %	Total= 40	16 (40%)	13 (32%)	11 (28%)

COMBINATION	DILUTION	CORRECT[a]	INCORRECT[b]	INDETERMINATE
Staph/Tor	1:32 v 1:64 (n=16)	6 (38%)	4 (25%)	6 (37%)
	1:64 v 1:128 (n=8)	6 (75%)	2 (25%)	0
	1:128 v 1:256 (n=8)	2 (25%)	6 (75%)	0
	1:256 v 1:512 (n=8)	4 (50%)	4 (50%)	0
Overall %	Total= 40	18 (45%)	16 (40%)	6 (15%)

[a] Lower dilution with higher kill [b] Higher dilution with higher kill.

The dilution data have been compared in order to demonstrate the presence or absence of a dose-response. It is also necessary to examine only the use-dilutions with an appropriate performance criterion to determine the disinfectant failure rate. The performance criterion in the Quantitative Carrier Test has been chosen as a reduction of >/= 99.999% of the original bacterial count. In terms of log reduction this would be >/= 5 logarithms. While the endpoint has been defined, it is not feasible to select an acceptable failure rate for all disinfectant classes based only on the performance of these four disinfectants. Additional testing with more disinfectants would be necessary to accurately determine pass/fail levels.

Using the chosen criterion, and examining the recommended use-dilutions, the rank of the tested disinfectants, from lowest to highest failure rate is as follows: 1) Vesphene IIse 2) Tergisyl 3) Tor and 4) Buckeye. (See Table 15). When all adjacent disinfectant dilutions in this study were examined for the percentage of incorrect, or unexpected results the failure rates were increased over those of the >/= 5 log criterion. This is to be expected since the method examined the performance of all dilutions, not just the use-dilution, and was intended to be a method of detecting a dose-response. The rank of the disinfectants, examining all dilutions, remained the same with the exception of Buckeye having a better performance than that of Tor. With both methods of analysis, the phenolic compounds perform better than the quaternary ammonium compounds.

The quaternary ammonium compounds, when tested against the gram-negative rods, did not perform well at the use-dilution and did not demonstrate dramatic dose-responses at dilutions eight times higher than the recommended use-dilution. The log reductions remained relatively constant throughout the dilutions. Against Staphylococcus aureus, the gram positive cocci, the quaternary ammonium compounds did quite well, with neither compound failing against this organism. This is consistent with previous work which demonstrated that quaternary ammonium compounds have greater activity against gram positive bacteria than gram negative bacteria.[43]

TABLE 15
FAILURE RATES WITH 99.999% KILL AT USE-DILUTION, ONE MINUTE EXPOSURE

DISINFECTANT	PERCENTAGE OF FAILURE
VESPHENE	0%
TERGISYL	5%
TOR	18%
BUCKEYE	28%

The exposure period for this study was one minute for both quaternary and phenolic compounds. This exposure was chosen in order to demonstrate the ability of the QCT to detect a dose-response over a series of dilutions. In real use situations, it is assumed that the exposure period would also approximate one minute. The contact time with the disinfectant is an important parameter in disinfectant testing. [9] The proposed actions of the quaternary compounds are inactivation of energy-producing enzymes, denaturation of cell proteins and cell membrane disruption. It is conceivable that the quaternary compounds' performance against the two gram-negative rods may have improved if the exposure had been lengthened. When S. aureus was tested with Buckeye at the use-dilution of 1:128 and 1:512 for periods of one and ten minutes, the only surviving growth occurred in the 1:512 dilution, one minute exposure replicates. The AOAC Use-Dilution Method was performed on the same day as a comparison. (See Table 16). At 1:128, one minute, there were two failures out of ten replicates, while the same dilution, held for ten minutes, had no failures. The 1:512 dilution, ten minute exposure, had only one failure versus nine failures for the one minute exposure period. The lengthened exposure would have been more meaningful if done with Pseudomonas aeruginosa since this organism is typically more resistant to disinfection than S. aureus.

The active ingredients in the quaternary ammonium compounds have been examined previously by the phenol coefficient method. (See Appendix) For n-alkyl (50% C14, 40% C12, 10% C16) dimethyl benzyl ammonium chloride the killing dilution was reported to be 1:45,000 for S. aureus with a 10 minute exposure. For didecyldimethyl ammonium chlorides, another active ingredient in Buckeye, the highest killing dilution for S. aureus was reported to be 1:63,000. For Tor, the active ingredient n-alkyl (60% C14, 30% C16, 5% C12, 5% C18) dimethyl benzyl ammonium chloride, the highest effective dilution was listed as 1:44,000.[43] However, it must be emphasized that these data are from the disinfectant manufacturers and have not been reliably documented by independent laboratories.[3] Based on the 99.999% kill criterion, the quaternary ammonium compounds examined in the QCT performed in a manner similar to those found in other independent research. On this basis it is concluded that the poor performance of the quaternary ammonium compounds is intrinsic to the compounds and the Quantitative Carrier Test accurately portrays this aspect.

TABLE 16

S. AUREUS VS BUCKEYE IN THE AOAC UDM

DISINFECTANT DILUTION	TIME (MINUTES)	FAILURES/REPLICATES
1:128	1	2/10
1:128	10	0/10
1:512	1	9/10
1:512	10	1/10

The performance of the phenolic compounds in the QCT was better than that of the quaternary ammonium compounds, reflecting the improved action of this class of disinfectants. Both phenolics, Tergisyl and Vesphene IIse, had excellent dose responses over doubling dilutions up to 16 times more dilute than the use-dilutions. If the endpoint for success was >/= 99.999% reduction of the starting inoculum, then Vesphene IIse had no failures, while Tergisyl had a 5% failure rate. This was much lower than the 18 and 28% failure rates of Buckeye and Tor, respectively. If the failures were examined over all dilutions, using the correct, incorrect or indeterminate criteria, then the failure rate rose to 11% for Vesphene IIse and 16% for Tergisyl. As with the quaternary compounds, the gram positive organisms, such as S. aureus, are more sensitive to the action of the phenolic compounds since they lack the additional outer cell membrane of the gram negative rods.[50] Both phenolics in this assay had use-dilution log reductions for S. aureus that were higher than those of the two gram negative rods.

The major difference between the two phenolics tested is that the active ingredients in Vesphene IIse are sodium phenates. These compounds, while more easily solubilized, are less effective than other phenol derivatives.[50] This apparently did not have a bearing in the QCT, since Vesphene IIse had no failures in this study. One explanation may be that the concentration of sodium phenates in Vesphene IIse is much higher than the active ingredients in Tergisyl. Vesphene IIse had 1405 ppm of phenolic derivatives while Tergisyl had 750 ppm of phenolic derivatives at its' use dilution.

This study has verified that the ultrasonic treatment and vortexing of the penicylinders is capable of removing all organisms surviving disinfectant immersion. Scanning electron microscopy of penicylinders, inoculated with S. aureus, revealed that no organisms remained on the surface after being treated in an ultrasonic waterbath and vortexer.

The use of Letheen as a neutralizing broth is superior for inactivating any disinfectant carryover, preventing bacteriostatic effects. The broth contains lecithin, which is a neutralizer and Tween 80, which acts as a lecithin dispersing agent. The original study of Letheen found that ten milliliters of Letheen broth was capable of neutralizing one milliliter of a 1:1500 dilution of a quaternary ammonium compound.[51] The QCT also utilized ten milliliter quantities of Letheen broth, with carryover being much

less than one milliliter. Therefore, disinfection beyond the one minute exposure time period should not have been a problem in this assay.

The filtrate of the Quantitative Carrier Test was sampled throughout one run of the assay and no organisms were recovered. This verified that surviving organisms were not being lost and were being recovered on the filter grid. Since survivors were recovered in the assay it was possible to determine if one organism or 100,000 organisms survived disinfectant exposure. The AOAC UDM can only assess growth or no growth and would not be able to detect this 5 log difference in numbers of surviving organisms. While complete kill is desired, it must be emphasized that working with biologic systems can be very difficult and some degree of error is inherent in any system. The advantage of the QCT over the AOAC UDM is that the magnitude of error is more accurately assessed.

Chapter 6

DISCUSSION AND CONCLUSIONS

The purpose of this study was to demonstrate that the Quantitative Carrier Test could distinguish between the performance of effective disinfectants, such as phenolics and ineffective disinfectants, such as the quaternary ammonium compounds. The QCT is a new method for testing disinfectants that is based on the framework of the AOAC Use-Dilution Method, especially the use of stainless steel penicylinders which provide a great challenge to disinfectants. The QCT, however, is an improvement over the AOAC UDM because the actual bacterial counts are obtained rather than observing tubes for mere presence or absence of growth. The log reduction, or percent kill, can be accurately determined using the QCT method. These results are reproducible and the degree of error present when working with a biologic system can be more accurately assessed. The ability of a disinfectant to kill organisms is dependent on factors such as the type of organisms present, the temperature of the assay, the period of exposure to the disinfectant and the starting bacterial inoculum.[4,8,11] Most factors can be controlled, with the exception of the inoculum, which can be held within certain ranges but cannot be precisely controlled. This is taken into account by using the log reductions as a standardizing method, while the AOAC UDM has no means of assessing different starting inoculums found in biologic systems.

The QCT presents disinfectants with a challenge and the design of the test insures that organisms that survive disinfectant exposure are recovered. The use of Letheen broth as a neutralizer prevents disinfectant carryover that would allow disinfection to extend past the allotted time period. Ultrasonic treatment and vortexing have been shown to remove survivors from the penicylinders and allow recovery on the filter grid. Sampling the filtrate has also demonstrated that surviving organisms are not lost during the filtration process.

This was a limited study to assess the feasibility of a new test method. The phenolics and quaternary ammonium compounds gave performances in the QCT which parallel their performances in previous disinfectant tests.[44-49] However, several additional areas of the Quantitative Carrier Test performance should be explored in the future. As an additional test of reproducibility it would be important to have several different laboratories test a series disinfectants using the QCT, similar to an earlier study by Rutala and Cole.[3] Further research should include a wider array of disinfectant classes to verify that results are similar and reproducible across different chemical formulations. The test has been shown capable of detecting dose-responses at one minute and an additional test would be further testing with longer exposure periods. Longer exposures, in line with the manufacturers' use recommendations would be more applicable for certifying disinfectants as effective or ineffective. After more extensive testing with different disinfectants and time periods it would be possible to define acceptable failure rates using the 99.999% kill endpoint. These parameters would be set for certification purposes to prevent the registration of ineffective disinfectants.

The Quantitative Carrier Test, on the basis of this study, is a useful test for investigating the action of disinfectants. The QCT is capable of distinguishing between effective and ineffective disinfectant classes. The QCT retains the framework of the sanctioned AOAC Use-Dilution Method, but is an improvement over the AOAC UDM because actual bacterial counts are obtained and log reductions can be accurately determined. This assay is a viable replacement for the sanctioned test since the results are reproducible and standardized, thus potentially improving the disinfectant registration process. Further testing with additional disinfectant classes and testing for interlaboratory variability may reinforce these preliminary conclusions concerning the Quantitative Carrier Test.

Chapter 7

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Appendix 1

EARLY DISINFECTANT TESTS

Many tests of disinfectant efficacy have been developed over the years but are no longer in use due to deficiencies. However, a few of the earliest tests are based on principles which are still valid. One of the earliest tests was developed prior to the discovery of the role of bacteria in food spoilage and disease. In 1750, Sir John Pringle developed a table of salt coefficients for the preservation of meat. Pringle put lean meat into separate glass jars with different salts. The endpoint was measured in a logical manner, by smell. Pringle made sea salt the standard and compared all other salts to it, assigning a numerical value to their efficiency.[17,18] (See Appendix)

In 1875 Bucholtz performed assays to determine the minimum inhibitory concentrations (MIC) of phenol, creosote, benzoic acid and salicylic acids. The MIC represents the lowest level of a drug or disinfectant that is bacteriostatic. If a substance is bacteriostatic it prevents the bacteria from multiplying but does not necessarily kill the organisms at that particular concentration. Bucholtz tested these disinfectants against mixed bacterial cultures which actually represented real-use situations.[19]

Robert Koch, a pioneer in the field of microbiology, developed the Silk Thread Test is 1881. This test simulated practical conditions by using silk threads impregnated with Bacillus anthracis spores. The threads were soaked for varying time periods in disinfectants including phenol and mercuric chloride. The threads were washed and then placed in nutrient media to recover any survivors. This assay had two distinct problems : 1) the threads provided protection for the spores and 2) the washing process did not remove all of the disinfectant, resulting in carryover into the nutrient media.[20]

In 1889 Geppert realized that the mercuric chloride was not being completely washed off the threads in the Koch test. The carryover was toxic to the surviving spores producing erroneous results. Geppert suggested the use of ammonium sulphide to neutralize the mercuric chloride. This was

the first use of a neutralizer in a disinfectant assay.[19]

The Rideal-Walker Assay

The Rideal-Walker test was formulated in 1903 and revised in 1921. In 1934, the test was adopted by the British Standards Institution as a qualitative suspension test of the relative activity of the phenolic derivatives of coal tar.[8] At the time this test was developed, there were three types of phenolics in wide use. The black fluids were basically coal tar solubilized with soap and were quite irritating if they came in contact with the skin. The white fluids were emulsified tar fractions which often left precipitates on surfaces. The clear soluble phenolics were inexpensive disinfectants such as lysol and sudol.[21,22]

The Rideal-Walker test was relatively simple in design. The original assay used a 24 hour old broth suspension of Salmonella typhi and standard dilutions of phenol, ranging from 1:95 to 1:115. Dilutions of the disinfectant were made with distilled water. Two hundred microliters of the bacterial suspension were added to five milliliters of the disinfectant dilutions. Subcultures were taken at 2.5, 5, 7.5, and 10 minutes using a standard loop to inoculate five milliliters of recovery broth. Recovery broths were incubated for 48 to 72 hours at 37° C. The endpoint was no visible growth in the subculture.[23] The phenol coefficient of the test was determined by dividing the dilution showing growth at 2.5 and 5 minutes but no growth at 7.5 minutes by the dilution of the phenol standard. The larger the resulting number, the more efficient the disinfectant in comparison to phenol.

The main advantages of the Rideal-Walker test were its relative simplicity, low cost, and speed of performance. In addition, the reproducibility of the assay was comparable to other existing disinfectant tests of the time.[23]

Although simple, the Rideal-Walker assay had many deficiencies that decreased its overall usefulness. When the test was first developed, Salmonella typhi was a significant public health hazard. Today, other organisms present much more of a hazard in hospital settings. Also, the use on only one organism could have allowed disinfectant manufacturers to formulate their product precisely to achieve high Rideal-Walker coefficients.[23] This problem was avoided in the 1980 Association of Official Analytical Chemists (AOAC) manual which required the use of Salmonella typhosa (ATCC 6539), Staphylococcus aureus (ATCC 6538) and Pseudomonas

aeruginosa (ATCC 15442) for the test.[24]

The use of distilled water as a diluent was also a deficiency as disinfectants in field situations were rarely diluted with distilled water. The disinfectants were not challenged by hard water components and so did not accurately reflect their true performance. Additionally, there was no organic matter present to deplete the disinfectant as there was in field situations. Finally, the recovery medium was not optimal for the survival of damaged bacterial cells.[23]

The desired endpoint of the assay was complete killing of the test organism in the broth recovery medium. While this could have occurred, it was also likely that the numbers of surviving organisms in the disinfectant broth were small and the chances of recovering the organisms on the transfer loop were extremely low. Therefore, the survivors could remain in the disinfectant and never be transferred to the recovery broth where they could be detected.[23]

Perhaps the greatest drawback of the Rideal-Walker test was not the procedure itself but the misuse of the test. The test was designed as a routine control check for phenolic compounds and not for other disinfectant classes. Errors arose when disinfectants other than phenolics were tested. Prickett, for example, found that the bactericidal efficacy of the quaternary ammonium compounds was overestimated because there was incomplete neutralization of the compounds during the assay.[25] Comparison of different disinfectant classes was not valid since phenol is a small, water soluble molecule while other preparations are larger, more complex, and contain emulsifying agents. Sykes suggested that a more realistic role for phenol is to verify that the test organisms are maintaining their intrinsic resistance to disinfectants.[5]

The Rideal-Walker test, although possessing the attributes of simplicity, rapidity and reproducibility, is obviously deficient in several major areas. It's use should continue to decline as the use of phenolic disinfectants declines. The assay is useful for testing the intrinsic resistant of test organisms and studying the protocol when developing other similar assays.

The Chick-Martin Test

The Chick-Martin test was developed as an improvement of the Rideal-Walker phenol coefficient test. It was devised in 1908 and originally introduced organic matter in the test in the form of 3% dried, sterilized human feces. Later, the feces were replaced by a 5% suspension of dried,

sterilized yeast.[23]

The Chick-Martin assay was in many ways similar to the Rideal-Walker assay. It utilized Salmonella typhi as the test organism which was inoculated into dilutions of the disinfectant and the reference standard, phenol. Both disinfectant dilutions contained 5% yeast suspensions as an additional challenge for the disinfectants. The contact time for the test was thirty minutes at 20° C. At the end of the test period, the broths were subcultured into duplicate recovery broths and incubated for 48 hours at 37°C.

Determination of the phenol coefficient was simple. The concentration of phenol which prevented growth in both subcultures was determined as was the concentration that permitted growth in both broths. The mean value was determined from these two concentrations. The procedure was repeated for the test disinfectant. If a negative and positive result was obtained, then this dilution value was used in the coefficient calculations. To obtain the final coefficient, the mean phenol value was divided by the tested disinfectant value.[26]

The Chick-Martin assay was not a great improvement over the earlier Rideal-Walker test. While the need for a challenge with organic matter was recognized, the actual test methodology was deficient in many of the same areas as Rideal-Walker. For example, the choice of Salmonella typhi as the test organism is not as important today as it was in 1908. Additionally, the chance of transferring surviving organisms into the recovery broths eventually reached a very low probability just as it did in the Rideal-Walker assay. This test is of little use today except to gain historical perspective on the field of disinfectant testing.

Appendix 2

CURRENT DISINFECTION ASSAYS

The Kelsey-Sykes Test

The Kelsey-Sykes disinfectant test is a capacity test that has undergone numerous revisions since its inception in 1969. A capacity test is a suspension test in which the activity of the disinfectant is challenged by repeated addition of a bacterial load.[27] According to Kelsey and Maurer, the test is intended for use by official laboratories or manufacturer's laboratories and is suitable for all types of disinfectants. Results obtained should serve as a guide for choosing effective concentrations of disinfectants used in hospital settings.[28]

The Kelsey-Sykes assay consists of two main parts: the selection of the organism most resistant to the test disinfectant and the determination of the concentration deemed most effective. The organisms involved in the initial selection process are Pseudomonas aeruginosa (NCTC 6749), Proteus vulgaris (NCTC 4635), E. coli (NCTC 8196), and Staphylococcus aureus (NTCC 4163). The organisms are subcultured daily into Wright and Mundy Broth (Bacto Synthetic Broth, AOAC Code No. 0352, Difco Ltd.) to which 10% sterile dextrose has been added. Between the fifth and fourteenth subculture the organisms are diluted 1:10 in Wright and Mundy broth. Pseudomonas aeruginosa must be filtered with Whatman's No. 4 filter paper before the dilution is made. Sets of ten doubling dilutions of disinfectant are made in Wright and Mundy broth with dextrose. To each 5 ml of diluted disinfectant 0.02 ml of the 1:10 organism dilution is added. The tubes are examined for 72 hours following incubation at 32^0 C -/+1^0 C to determine which organism is able to grow in the disinfectant dilutions. This organism will present the disinfectant with the best challenge in the second part of the assay.[28]

An important aspect of the assay is its challenge of the disinfectant with both clean and dirty conditions. The organic matter in this assay is a yeast suspension. The dirty inoculum is prepared by suspending yeast in hard

water while the test organism is inoculated into 10 ml of Wright and Mundy broth and incubated for 24 hours prior to the test. To achieve the final 2% concentration of yeast, six ml of the organism suspension is added to four ml of the yeast suspension. The suspension must contain 10^8 to 10^{10} viable organisms per milliliter and the ten milliliters is adequate to test three disinfectant concentrations.

The clean inoculum is prepared by inoculating 10 ml of Wright and Mundy broth with the organism and incubating for 24 hours. The suspension is centrifuged for 15 minutes at 3000 rpm and the organism pellet is resuspended in 10 ml of sterile hard water. Again, the Pseudomonas aeruginosa must be filtered prior to centrifugation. The hard water is specified by the World Health Organization to be 342 ppm hardness. The formulation requires 0.304 g anhydrous calcium chloride and 0.139 g magnesium chloride hexahydrate per liter of distilled water. Once again, the final organism suspension must contain between 10^8 to 10^{10} organisms per milliliter.

Three disinfectant concentrations are tested against the resistant organism chosen in the first portion of the assay. Concentration B is the disinfectant concentration which is expected to pass the test. Concentration A is B less 50%, while Concentration C is B plus 50%. The dilutions must be freshly prepared on the day of the test in sterile hard water. The actual assay is performed at 20-22^0 C. There are three tubes labeled A, B and C, each containing 3 mls of the particular disinfectant. One milliliter of the bacterial inoculum is added at 0, 10 and 20 minutes into the assay. At 8, 18 and 28 minutes, 0.02 ml is subcultured into each of five recovery broths. The recovery broths are incubated for 48 hours at 32^0 C and examined for evidence of growth.

A particular disinfectant concentration is approved if it demonstrates no growth in at least two of the five recovery broths in the sets from the eight minute and eighteen minute subcultures. The 28 minute recovery broths are not used in determinations and Kelsey gives no explanation for their inclusion in the test. A concentration must pass the test on three separate occasions, each requiring fresh inoculum and disinfectant dilutions. The initial disinfectant concentration (A,B, or C) is the one deemed as passing, although the actual concentration is decreased with each bacterial addition. Kelsey and Maurer state that this method provides a safety margin for in-use

situations.[28]

The Kelsey-Sykes test is in worldwide use in places such as the United Kingdom, South Africa, Australia and Malaysia.[29] The advantages of this sanctioned test include the attempt at providing realistic conditions by including yeast as organic matter, using organisms found in the hospital environment, and testing the most resistant organisms available. Additionally, the recovery broth is a nutrient broth containing Tween 80, which is an effective neutralizing agent to reduce disinfectant carryover.[23]

The Kelsey-Sykes test, like all disinfectant tests, has inaccuracies and disadvantages. These problems range from those which cause inconveniences, to those which may result in inaccurate results. The most obvious fault of the test is that it is complicated. Assays of this nature are therefore less reproducible in laboratories which do not routinely perform the assay to maintain a level of competency. One reason the test is technically difficult is the insistence by the developers that the third bacterial inoculum be used. The results of this third addition are not used in the final calculations and seem to be an unnecessary portion of the test.[23]

Certain components of the assay have also been cited as areas of concern. For example, the use of yeast as organic matter may not be optimum. One study by Charley and Harter found that as the age of the stored yeast increases, it presents less of a challenge to certain disinfectants. They found the concentrations of iodophors necessary to pass the test decreased as the yeast age increased.[30] This may also be true of other disinfectant classes .

The requirement of WHO hard water as a diluent for organism and disinfectant suspensions is an inconvenient and perhaps unnecessary requirement. The exact role of hard water in field situations is not known. Croshaw states that the presence of hard water may actually affect the activity of some disinfectants. However, the use of WHO hard water is an attempt to standardize the procedure and may present a challenge whose benefits outweigh the additional constraints placed on the laboratories.

An additional problem sited by Croshaw is the lack of a reference standard, or control disinfectant, to insure that the chosen test organism is performing as expected. Without a control, there is no assurance that the organisms' intrinsic resistance has not been altered by the repeated broth subcultures.

In the 1974 revision of the test, Kelsey and Maurer stated that the Kelsey-Sykes test was suitable for testing all types of disinfectants: alcohols, aldehydes, ampholytes, diguanides, hypochlorites, iodophors, phenolics and quaternary ammonium compounds.[28] Others have presented data which disputes this statement. Coates found the test to be ill-suited for the testing of hypochorite disinfectants since they are inactivated by the presence of organic matter. Of course, this is a problem in field situations, but Coates suggests that the use of such a complex test is unnecessary. The true activity of hypochlorites can be assessed by the levels of free chlorine present and the hydrogen ion concentration.[30]

Cowen, in a later study, tested iodophors, quaternary ammonium compounds, phenols and hypochlorites with the Kelsey-Sykes methodology in several laboratories. Using reproducibility as an endpoint, Coates concluded that the test was suitable for testing phenolic compounds and suggested that changes in the test would be needed before the assay could be used for all disinfectant classes.[31]

The Kelsey-Sykes test is a capacity test which seeks to determine the most effective concentrations of disinfectants use in hospital settings. While it is useful to test the performance of the disinfectant when challenged by organic matter, it seems that the assay could be revised in such a manner as to simplify the process and still provide the user with meaningful results. The major area in which improvement is needed is in test reproducibility. Still, the assay is a vast improvement over earlier tests such as the Rideal-Walker and Chick-Martin tests.

Appendix 3

METHODS AND MATERIALS OF THE AOAC USE-DILUTION METHOD

Culture Media:

1) Nutrient broth: Combine 5 g NaCl, 5 g beef extract (Difco) and 10 g peptone (Anatone, American Laboratories, Inc.) in 1 liter of H_2O and boil for 20 minutes. Adjust to pH 6.8, filter and place 10 ml aliquots in 20 x 150 mm test tubes. After autoclaving for 20 minutes at 121^0 C, the broth is used for daily subculturing of test organisms.

2) Synthetic broth: Solution A: Dissolve 0.05 g L-cystine, 0.37 g DL-methionine, 0.4 g L-arginine.HCl, 0.3 g DL-histidine.HCl, 0.85 g L-lysine.HCl, 0.21 g L-tyrosine, 0.5 g DL-threonine, 1.0 g DL-valine, 0.8 g L-leucine, 0.44 g DL-isoleucine, 0.06 g glycine, 0.61 g DL-serine, 0.43 g DL-alanine, 1.3 g L-glutamic acid.HCl, 0.45 g L-aspartic acid, 0.26 g DL-phenylalanine, 0.05 g DL-tryptophan, and 0.05 g L-proline in 500 mL H_2O containing 18 mL 1N NaOH.

Solution B: Dissolve 3.0 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 1.5 g KH_2PO_4, 4.0 g Na_2HPO_4, 0.01 g thiamine.HCl, and 0.01 g nicinamide in 500 mL H_2O.

Solutions A and B are mixed and placed in 10 mL aliquots in 20 x 150 mm tubes and autoclaved for 20 minutes at 121^0 C. Add 0.1 mL of sterile 10% glucose to each tube and grow test organisms with tube slanted 8^0 from horizontal.

3) Nutrient agar: To either nutrient broth or synthetic broth, add 1.5% Bacto agar (Difco) and adjust pH to 7.2-7.4. Autoclave and slant for cooling.

Subculture Media:

Subculture media is chosen depending on which disinfectant class is being tested. The media should adequately neutralize the disinfectant and provide a satisfactory medium for recovery of surviving bacterial cells.

1) Nutrient broth: see above.
2) Fluid thioglycolate medium USP XX: In 1 liter H2O mix 15.0 g pancreatic digest of casein, 5.0 g H_2O-soluble yeast extract, 5.5 g glucose.H_2O, 2.5 g NaCl, 0.75 g agar and 0.5 g L-cystine. Heat to dissolve, then add 0.5 g Na thioglycolate or 0.3 g thioglycolic acid. Adjust the pH to 6.9-7.3 with 1N NaOH. Add 1.0 mL 0.1% Na resazurin solution and dispense in 10 mL aliquots to 20 x 150 mm tubes. Autoclave for 20 minutes at 121° C. Immediately cool to 25°C and protect from light. Use for subculturing when disinfectants contain oxidizing products or heavy metals.
3) Letheen broth: In 400 mL hot H_2O, mix 5.0 g polysorbate 80 and 0.7 g lecithin (Azolectin, Associated Concentrates) and boil until clear. Add 600 mL of solution of 5.0 g beef extract, 10.0 g peptone and 5.0 g NaCl in H_2O and boil 10 minutes. Adjust pH to 6.8-7.2 with 1N NaOH and/or 1N HCl. Filter and aliquot 10 mL portions to 20 x 150 tubes and autoclave. Use as a recovery medium when the disinfectant contains cationic surface active materials, such as quaternary ammonium compounds and phenols.
4) Cystine trypticase agar(BBL): Dissolve 29.5 g in 1 L H_2O by boiling for approximately one minute. Dispense 10 mL aliquots into 20 x 150 mm tubes and autoclave for 15 minutes at 12 lb pressure. Store at 20-30° C and use for monthly subculture of Pseudomonas aeruginosa.
5) Other subculture media: To fluid thioglycolate medium, add 0.7 g lecithin and 5.0 g polysorbate 80. Protect from light.

Apparatus and Reagents:

1) Transfer loop: Using No. 23 gage platinum wire, make a 4 mm interior diameter loop on 50-75 mm length wire. A 4 mm loop may be obtained from Matthey-Bishop, Inc and fused onto a suitable length of wire. Loop should be at a 30^0 angle with the wire.

2) Test organisms: Salmonella choleraesuis (ATCC 10708). Maintain stock culture on nutrient agar slants, subculturing monthly. Store transfer at 2-5° following 2 days incubation at 37° C.
Staphylcoccus aureus (ATCC 6538).
Pseudomonas aeruginosa (ATCC 15442). Maintain stock culture on BBL Cystine Trypticase Agar slants. Subculture monthly, storing at 5° following 48 hour incubation.
3) Phenol stock solution: Place 50g USP Phenol into a beaker. Dissolve in H_2O and wash into 1 liter flask, dilute to volume. Transfer 25 mL of stock solution into a 500 mL volumetric flask and dilute to volume with H_2O. Then transfer 15 mL of this solution to a 500 mL flask and add 30 mL of the standard KBr-$KBrO_3$ solution. Add 5 mL HCl and insert stopper. Shake flask frequently for 30 minutes and allow to stand for 15 minutes. Quickly add 5 mL of 20% KI solution and immediately replace stopper. Mix well, remove stopper and rinse it and neck of flask with a small amount of H_2O. Titrate with 0.1N $Na_2S_2O_3$ using starch indicator. Percentage of phenol in stock solution = (30 - mL 0.1N $Na_2S_2O_3$ solution for titration) x 0.001569 x 1333 x 100/1000. If necessary to adjust the solution, add H_2O or phenol to the mixture and protect from light.
4) Potassium bromide-bromate solution: Transfer 30 mL to a flask and add 25 mL H2O, 5 mL 20% KI solution and 5 mL HCl. Shake and titer with 0.1N $Na_2S_2O_3$ using starch indicator.

Phenol Coefficient Method

Make a 1% stock dilution of the substance to be tested and make final dilutions in from this stock into test tubes. From the 5% stock phenol solution make a 1:90 and 1:100 dilution into test tubes. Each tube should contain 5 mL of each dilution. Place the dilution tubes and the tube containing the test organism in the 20^0 water bath and allow to equilibrate for five minutes. Add 0.5 mL of the test organism to each dilution at the appropriate time interval. If ten tubes are being used, then 30 seconds should elapse between tubes and an additional 30 seconds should elapse before the subculture begins. Thus, after five minutes exposure to the disinfectants, subculture one loopful to a

subculture broth and repeat at ten minutes and 15 minutes.

In order to properly inoculate the disinfectant tubes, they must be held in a slanting position and the pipet containing the organism must be inserted above the disinfectant without touching the suspension. The tubes must be agitated after organism addition to insure an even suspension. For the subculture, the tubes should be held at a 60^0 angle and the loop should be withdrawn so that the plane of the loop is parallel to the surface of the liquid. The loop and each test tube mouth should be flamed before every transfer, and the loop should not be allowed to touch the sides or the mouth of the test tubes.

All subculture tubes should be thoroughly mixed after inoculation. The subculture tubes are incubated at 37^0 for 48 hours. Examine macroscopically for growth.

Calculations: Determine the highest dilution killing the test organism in 10 minutes but not 5 minutes and divide the numerical value of the this dilution by the greatest dilution of phenol killing the organism in 10 minutes but not 5 minutes. The phenol coefficient should be calculated to the nearest 0.1.

Results: For Salmonella typhi the following results should be obtained:

Phenol	5 Minute	10 Minute	15 Minute
1:90	+ or 0	+ or 0	0
1:100	+	+	+ or 0

For Staphylococcus aureus the following results should be obtained:

Phenol	5 Minute	10 Minute	15 Minute
1:60	+ or 0	+ or 0	0
1:70	+	+	+

For Pseudomonas aeruginosa the following results should be obtained:

Phenol	5 Minute	10 Minute	15 Minute
1:80	+ or 0	+ or 0	0
1:90	+	+	+

If none of the dilutions of the disinfectant shows growth in the 5 minute broth and killing in the 10 minute broth then a dilution must be estimated only when 3 consecutive dilutions give the following results: 1) no growth in 5 minutes, 2) growth in 5 and 10 minutes but not in 15 minutes and 3) growth in 5, 10, and 15 minutes. An example follows:

Disinfectant	5 Minute	10 Minute	15 Minute
1:300	0	0	0
1:350	+	+	0
1:400	+	+	+
Phenol 1:90	0	0	0
Phenol 1:100	+	+	0

The phenol coefficient would be calculated as:

$$325/95 = 3.4$$

The Use-Dilution Method

Organisms: 1) Salmonella choleraesuis (ATCC 10708) should be inoculated into a tube of nutrient broth and incubated at 37^0. Complete three consecutive daily transfers. Prior to the actual test, inoculate two tubes of nutrient broth for each ten carriers to be tested with one loopful of the organism suspension. These should be 48 to 54 hours old at the beginning of the assay.

2) A 48 to 54 hour old suspension of Staphylococcus aureus (ATCC 6538).

3) Pseudomonas aeruginosa (ATCC 15442) should be maintained on BBL cystine trypicase agar stored at 5^0 and transferred every 30 days. Nutrient broth suspensions should be transferred daily for 30 days, incubating at 37^0. A 48 to 54 hour suspension should be used for the assay after the pellicle has been removed. Twenty milliliters of the

culture are required for inoculation of 20 penicylinders.

Asparagine solution: Make a stock supply of 0.1% asparagine solution in H_2O in a flask and autoclave for 20 minutes at 121^0.

Sodium hydroxide: Maintain a 4% solution for cleaning the penicylinders prior to use.

Transfer loops and needles: See phenol coefficient method. The needle should have a 3 mm right angle bend at the end of a 50-75 mm nichrome wire, No 18 B&S gage.

Penicylinders: From S&L Metal Products Corp., Maspeth NY, obtain type 304 stainless steel penicillin cups. Measurements should be: 7-9 mm outer diameter, 5-7 mm inner diameter, 9-11 mm in length.

Petri dishes: Obtain sterile petri dishes matted with 2 layers of Whatman No. 2, 9 cm filter paper.

Procedure of the AOAC UDM

The penicylinders should be stored overnight in 1N NaOH and rinsed with tap water until the washoff is of neutral pH. Following this, rinse the carriers twice with distilled water and place the cylinders in groups of ten into 25 x 150 mm test tubes; cover with the asparagine solution. The tubes should be autoclaved for 20 minutes at 121^0 C. Using a flamed nichrome wire hook, aseptically transfer 20 of the penicylinders into 20 mL of a 48 to 54 hour old suspension of the organism being tested. Allow the cylinders to remain in the suspension for fifteen minutes, then remove with the flamed hook and place the cylinders vertically in the sterile petri dish matted with filter paper. Cover the dish and place in an incubator at 37^0 for at least 20 minutes but no more than 60 minutes. The remaining broth suspension is reserved for use in the phenol coefficient portion of the assay. (See above).

Prepare the dilutions of the disinfectant to be tested in sterile distilled water. Add one contaminated cylinder to each of the ten tubes of

diluted disinfectant at one minute intervals. Exactly ten minutes after the immersion of the first cylinder, begin transfer of the cylinder to an individual subculture broth. The transfer hook should be flamed and cooled before each transfer and all excess disinfectant should be allowed to drain before placing in the subculture broth. The tops of all test tubes should be flamed prior to entry and the tubes should be swirled three times before being placed back into the 20^0 water bath. Immediately after transfer of all cylinders, place the tubes in a 37^0 incubator and incubate for 48 hours. Each tube should be recorded as growth or no growth upon visual inspection.

If lack of disinfectant neutralization is suspected, transfer each cylinder to a new tube of sterile medium and reincubate for 48 hours. If the disinfectant is such that it adheres to the cylinder surface, as may occur with concentrated acids and alkalies and wax emulsions, then it is necessary to transfer each cylinder to a new subculture broth 30 minutes after the initial transfer. Incubate both tubes for 48 hours.

There should be no growth on the ten cylinders in order to confirm the use-dilution concentration. If there are failing tubes it is necessary to repeat the test using higher concentrations of the disinfectant. *Note*: While killing in 10 of 10 replicates provides a reasonably reliable index in most cases, killing in 59 of 60 replicates is necessary to achieve the confidence level of 95%.

From: *Official Methods Of Analysis (1984), 14th ed. AOAC, Arlington VA, Chapter4, Disinfectants pp 65-68.*

Appendix 4

METHODS AND MATERIALS OF THE QUANTITATIVE CARRIER TEST

Culture media and Reagents

Nutrient Broth: In 1000 ml of sterile water (Baxter) dissolve 8 grams of Bacto nutrient broth (Difco Laboratories, Detroit MI,48232). Heat to dissolve, dispense in 10 ml aliquots into 25 x 150 mm tubes. Autoclave for 15 minutes at 121^{0}C.

Letheen Broth: In 1000 ml of sterile water (Baxter) dissolve 25.7 grams of Bacto Letheen Broth (Difco). Heat to boiling, dispense in 20 ml quantities into 25 x 150 tubes. Autoclave for 15 minutes at 121^{0}C.

Letheen Agar: In 1000 ml of sterile water (Baxter) dissolve 32 grams of Bacto Letheen Agar (Difco). Boil to dissolve completely. Autoclave for 15 minutes at 121^{0}C and then dispense in 19 ml quantities into 20 x 100 mm sterile petri dishes.

Tween 80 Saline: 1 ml polyoxyethylene (20) sorbitan monooleate (J.T. Baxter Chemical Co.) in 1000 ml of 0.9% Sodium chloride, USP (Travenol Laboratories Inc., Deerfield IL 60015). Autoclave for 15 minutes at 121^{0}C and dispense in 10 ml quantities into plastic tubes. Additionally, dispense 99 mls into glass bottles and autoclave for 15 minutes at 121^{0}C.

Sterile Water for Irrigation: Baxter Healthcare Corporation
Deerfield, IL 60015

Asparagine: Dissolve 1 gram Bacto Asparagine (Difco) in 1000 ml of sterile water (Baxter). Autoclave for 15 minutes at 121^{0}C.

Phenolphthalein: 0.05 grams of phenolphthalein (Fisher) dissolved in 50 ml sterile water and 50 ml 95% ethanol.

1N Sodium hydroxide: Sigma

Disinfectants

1) Sodium Xylene Sulfonate 10.8%, Triethanolamine Dodecylbenzene Sulfonate 6.3%, o-Phenylphenol 5.7%, Trisodium Ethylene Diamine Tetracetate 3.0%, p-tert-Amylphenol 1.8%, Inert ingredients 72.4% (including detergent, other cleaning agents and no phosphorus compounds). Use dilution is 1:100.
Lehn & Fink Industrial Products Division
Montvale, NJ 07645

2) Sodium o-phenylphenate 9.65%, sodium p-tertiary amylphenate 8.34%, inert ingredients 82.01%. Use dilution is 1:128.
Vestal Labs
St. Louis, MO 63110

3) Didecyl dimethyl ammonium chloride 9.22%, n-Alkyl (C_{14} 50%, C_{12} 40%,C_{16} 10%) dimethyl, benzyl ammonium chloride 6.14%, inert ingredients 84.64%. Use dilution is 1:256.
Buckeye International, Inc.
Maryland Heights, MO 63043

4) n-Alkyl (C_{14} 60%,C_{16} 30%,C_{12} 5%,C_{18} 5%) dimethyl benzyl ammonium chloride1.6%, n-Alkyl (C_{12} 50%,C_{14} 30%,C_{16} 17%, C_{18} 3%) dimethyl ammonium chlorides 1.6%, inert ingredients 96.8%. Use dilution is 1:64.
Huntington Labs, Inc.
Huntington, IN 46750

All disinfectant dilutions were prepared with sterile, distilled water at the manufacturer's recommended use dilution. Additional dilutions were prepared above and below the use-dilution . Dilutions were

aliquoted in 10 ml quantities into 25 x 150 mm tubes and brought to 20^{0}C in a circulating water bath. The pH of each dilution was recorded for each run.

Test Organisms

Salmonella cholerasuis (ATCC 10708)
Staphylococcus aureus (ATCC 6538)
Pseudomonas aeruginosa (ATCC 15442)

Organisms were obtained from the American Type Culture Collection (Rockville, MD 20852) and stored at -70^{0}C prior to use. After thawing, the organisms were subcultured to sheep blood agar five days prior to the test. Subsequent subcultures were in nutrient broth. Organisms were subcultured daily for not more than 30 days. Broth subcultures used in the assay were 48-54 hours old.

Penicylinders

Type 304 stainless steel penicillin cups, dull finish. S & L Metal Products Corporation, Maspeth, NY. Penicylinders were stored in IN NaOH overnight. Prior to the assay the penicylinders were rinsed with tap water until they tested neutral with phenolphthalein. They were then rinsed twice with distilled water, drained, covered with 0.1% asparagine and autoclaved for 15 minutes at 121^{0}C.

Apparatus

Circulating waterbath: Haake, Saddle Brook, NJ 07662
LED thermometer (Fisher)
Volumetric flasks
Wire hook: Nichrome wire with a 3 mm right angle hook
Sterile petri dishes lined with one sheet of Whatman's No. 2 filter paper.
Timer
Vortexer: Vortex-Genie, Fisher Scientific, Springfield, MA

Ultrasonic Water Bath: Health-sonics Corp. Pleasanton, CA
pH meter
Stereoscope
Erlenmeyer flask
Magnetic stir bar
Hot plate stirrer: Corning
Test tube racks
25 x 150 mm glass test tubes
Polystyrene culture tubes, 16 x 125 mm, Fisher Scientific, Pittsburgh, PA
Filter flask
Filters: QA Laboratories Ltd
135 The West Mall
Toronto, Canada M9C IC2
Filter grids: 0.45u ISO-GRID 100, QA Laboratories Ltd.