USE-DILUTION CONFIRMATION TESTS FOR RESULTS OBTAINED BY PHENOL COEFFICIENT METHODS*

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It has been commonly accepted that germicides used at dilutions equivalent in efficiency against *S. typhosa* to 5 per cent phenol at 20°C. in the phenol coefficient method will possess reasonable margins of safety for the destruction of infective agents likely to be the object of most general disinfection processes. The conventional method of arriving at the maximum safe use-dilution presumed to be equivalent in efficiency to 5 per cent phenol in the method is to multiply the phenol coefficient number found by the figure 20 to determine the number of parts of water in which 1 part of product is to be incorporated. While there may be considerable reason to doubt that dilutions of the various types of commercial germicides made up according to this formula do have germicidal efficiencies equivalent to 5 per cent phenol, this procedure has, with certain types of products in the past, provided for a reasonable margin of safety for disinfecting floors, walls, equipment, and facilities from which most extraneous organic matter had been removed. This has been pointed out by Varley and Reddish (1) and Reddish (2), and was confirmed many times in the laboratory of the Insecticide Division with commercial samples.

However, the cleaning processes of floors, walls, and certain equipment are often of a superficial nature and cannot be relied upon to reduce the amount of interfering organic matter or the number of bacteria to very low levels. During the last 10 years a rather alarming increase has been noted in the number of commercial products which, under these conditions, do not provide adequate margins of safety for disinfection even though they bear apparently valid phenol coefficient claims. It should be made a matter of record that most of these products will disinfect surfaces at the dilution indicated to be safe by the phenol coefficient number if they are applied after very thorough cleaning operations, but ordinary practices in janitorial services, home and farm sanitation programs, and even in hospital maintenance schedules cannot be relied upon to provide floor, wall, and fixed equipment surfaces of sufficient cleanliness to assure this result.

It appeared necessary, therefore, to develop some confirmatory test procedures which could be employed as a check on the practical significance of phenol coefficient values. Use-dilution testing of disinfectants was proposed by Mallman and Hanes (3) in 1944, and a procedure of this

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type was compared directly with the phenol coefficient method by Mallman and Leavitt (4) in 1948. As a result of these comparisons, it was reported that techniques of this kind provide a more dependable index to the actual safe use-dilution in the field than the phenol coefficient test, particularly with those products that exhibit high phenol coefficients.

The use-dilution method described by Mallman and Leavitt and numerous modifications of this method have been studied over a 5-year period in which many direct comparisons have been made on commercial germicides of all types. The results checked with those obtained in the phenol coefficient method and in tests conducted under conditions of actual use. In these studies particular attention has been given to the development of a procedure of sufficient precision to warrant acceptance for referee work, and to the accuracy of the end result in terms of actual disinfecting value.

Out of these studies, use-dilution methods have been developed for the specific purpose of confirming phenol coefficient values. As was found with the Mallman and Leavitt method, the results appear to provide more dependable indices to actual safe use-dilutions in the field than the phenol coefficient test, particularly with those products compounded from chemicals which are germicidally active at high dilutions. Also, collaborative test data have been obtained which indicate that they have sufficient precision to justify acceptance for referee purposes.

METHOD I

(Using *Salmonella cholerasuis*)

REAGENTS

(a) *Culture media.*—**(1)** *Nutrient broth.*—Boil 5 g beef extract (Difco), 5 g NaCl, and 10 g Armour peptone (quality specially prepared for disinfectant testing) in 1 l H_2O 20 min., adjust to *p*H 6.8 and make to vol. with H_2O. Filter thru paper, place 10 ml quantities in 20×150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lb pressure for 20 min.

(2) *Nutrient agar.*—Dissolve 1.5% Bacto agar (Difco) in nutrient broth and adjust to *p*H 7.2–7.4; place 15 ml quantities in 25×150 mm tubes, plug with cotton, sterilize at 15 lb pressure for 20 min., slant, and allow to solidify at room temp.

(3) *Subculture media.*—Use (a), (b), or (c), whichever gives lowest result:

(a) Nutrient broth described in **(a)(1)**.

(b) *Fluid thioglycollate medium U.S.P. XIII.*—Mix 0.75 g l-cystine, 0.75 g agar, 2.5 g NaCl, 5.5 g dextrose, 5.0 g H_2O-sol. yeast ext., 15.0 g pancreatic digest of casein with 1 l H_2O; heat to dissolve on H_2O bath, add 0.5 g Na thioglycollate or 0.3 g thioglycollic acid, and adjust with *N* NaOH to *p*H 7.0±0.1; reheat without boiling and filter thru moistened filter paper; add 1.0 ml freshly prepd 0.1% Na resazurin soln; tube in 10 ml quantities in 20×150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lb steam pressure 20 min.; cool at once to 25° and store at 20–30°.

(c) *Letheen broth.*—Dissolve 0.7 g lecithin (azolectin) and 5.0 g sorbitan monooleate ("Tween 80") in 400 ml hot H_2O and boil until clear; add 600 ml aq. soln of 5.0 g beef extract (Difco), 10.0 g peptone (Armour), and 5 g NaCl, and boil 10 min.; adjust with *N* NaOH and/or *N* HCl to *p*H 7.0±0.2 and filter thru coarse

filter paper; tube in 10 ml quantities in 20×150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lb pressure 20 min.

With oxidizing products and products formulated with toxic compounds contg certain heavy metals like Hg, (b) will usually give the lowest result. With products contg cationic surface active materials, (c) will usually give lowest results.

(b) *Test organism, Salmonella cholerasuis* (A.T.C.C. 10708).—Carry stock culture on nutrient agar slants. Transfer once a month and incubate new stock transfer 2 days at 37°, then store at room temp. From stock culture inoculate tube of nutrient broth and incubate at 37°. Make 3 consecutive 24 hr transfers, then inoculate tubes of nutrient broth (2 for each 10 carriers to be tested) using one loop of inoculum with each tube, and incubate at 37° for 44–48 hrs.

(c) *Phenol.*—Use phenol, U.S.P., which has congealing point 40° or above. Use 5% soln as stock soln and keep in well-stoppered amber bottles in relatively cool place, protected from light. Standardize with 0.1 *N* K or Na bromide-bromate soln, **39.18.**

(d) *Sterile distilled water.*—Prep. stock supply distd H_2O in 1 l flasks, plug with cotton, sterilize at 15 lb pressure for 20 min. and use to prep. dilns of medicants.

(e) *Asparagine.*—Make stock supply of 0.1% soln of asparagine ("Bacto") in distd H_2O in Erlenmeyer flasks of convenient size, plug with cotton, and sterilize at 15 lb for 20 min. Use to cover metal carriers for sterilization and storage.

(f) *N* NaOH.—Maintain stock supply of NaOH soln of ca *N* (4%) for cleaning metal carriers prior to use.

APPARATUS

(a) *Glassware.*—1, 5, and 10 ml volumetric pipets; 1, 5, and 10 ml Mohr pipets graduated to 0.1 ml or less; 100 ml stoppered cylinders graduated in 1 ml divisions; Pyrex lipped test tubes 25×150 mm; straight side Pyrex test tubes 20×150 mm; 15×110 mm Petri dishes, 100 ml, 300 ml, and 1 l Erlenmeyer flasks. Plug all tubes and flasks with cotton. Sterilize all glassware 2 hrs in hot air oven at 180° employing closed metal containers for pipets and Petri dishes.

(b) *Water bath.*—Insulated relatively deep H_2O bath with cover having at least 10 well spaced holes which admit medicant tubes but not their lips.

(c) *Racks.*—Any convenient style. Conventional wire racks or blocks of wood with deep holes are satisfactory. Have holes well placed to insure quick manipulation of tubes.

(d) *Transfer loops and needles.*—(1) Make 4 mm (inside diam.) single loop at end at 2–3 inch Pt alloy wire No. 23 B&S gauge. Have other end in suitable holder (glass or Al rod). Bend loop at a 30° angle.

(2) Make 3 mm right angle bend at end of 2–3 inch nichrome wire No. 18 B&S gauge. Have other end in suitable holder (glass or Al rod).

(e) *Carriers.*—Polished stainless steel cylinders (penicillin cups)* with an outside diam. of 8±0.1 mm.†

(f) *Petri dishes.*—Have ca 6 sterile Petri dishes matted with a layer of S&S No. 597, 9 cm filter papers.

DETERMINATION

Soak ring carriers overnight in *N* NaOH soln, rinse with tap H_2O until rinse H_2O gives neutral reaction to phenolphthalein, then rinse 2 times with distd H_2O; place cleaned ring carriers in multiples of 10 in cotton plugged Erlenmeyer flasks or 25×150 mm cotton plugged Pyrex test tubes, cover with 0.1% soln of asparagine (e), sterilize at 15 lb for 20 min., cool and hold at room temp. Transfer 20 sterile ring carriers using flamed nichrome wire hook into 20 ml of a 44–48 hr nutrient broth

* See Federal Register, Vol. 12, No. 67, p. 2217, April 4, 1947.

† (May be purchased from Erickson Screw Machine Products Co., 25 Lafayette Street, Brooklyn, New York.)

test culture in a sterile 25×150 mm medicant tube. After 15 min. contact period, remove cylinders, using flamed nichrome wire hook, and place on end in vertical position in a sterile Petri dish matted with filter paper. Place in incubator at 37° and allow to dry for no less than 10 min. and no more than 60 min. Hold the broth culture for dctn of its resistance to phenol by the phenol coefficient method.

From the 5% stock soln make 1–90 and 1–100 dilns of the phenol directly into medicant tubes. Place tube for each diln in H_2O bath and allow to come to temp. Make stock soln of the germicide to be tested in sterile glass stoppered cylinder. From this soln make 10 ml dilns to be tested depending upon the phenol coefficient found and/or claimed against *S. typhosa* at 20° directly into each of ten 25×150 mm medicant tubes, and then place the 10 tubes in the H_2O bath at 20° and allow to come to temp. Det. the diln to be tested by multiplying the phenol coefficient number found and/or claimed by 20 to det. the number of parts of H_2O in which one part of germicide is to be incorporated.

Add 0.5 ml of the test culture suspension to the 1–90 diln of the phenol control; after a 30 sec. interval, add 0.5 ml to the 1–100 diln of the control, using sterile cotton plugged pipets. After adding culture, agitate tubes gently but thoroly to insure even distribution of bacteria and replace in bath; 5 min. after seeding first medicant tube, transfer 1 loopful of mixt. of culture and dild phenol from medicant tube to corresponding subculture tube. At end of 30 sec. interval, transfer loopful from second medicant tube; 5 min. after making first set of transfers begin second set of transfers for 10 min. period; and finally repeat for 15 min. period. Use technique of loop sampling, flaming loop and mouths of tubes and agitating medicant and subculture tubes as outlined in phenol coefficient method. Incubate subcultures at 37° for 48 hrs and read results. Resistance in the 44–48 hr culture of *S. cholerasuis* should fall within range specified for the 24 hr culture of *S. typhosa* in the phenol coefficient method.

Add one contaminated dried cylinder carrier to each of the 10 tubes of the use-diln of the germicide to be tested at 1 min. intervals. Thus, by the time the 10 tubes have been seeded, 9 min. will have elapsed plus a 1 min. interval before transfer of the first carrier in series to an individual tube of subculture broth. This interval is a constant for each tube with the prescribed exposure period of 10 min. The 1 min. interval between transfers allows adequate time for flaming and cooling nichrome wire hook and making transfer in a manner so as to drain all excess medicant from carrier. Flame lips of medicant and subculture tubes in conventional manner. Immediately after placing carrier in the medicant tube, swirl tube 3 times before placing it back into bath. Shake subculture tubes thoroly, incubate 48 hrs at 37°, and report results as + (growth) or − (no growth) values. Where there is reason to suspect that lack of growth at the conclusion of incubation period may be due to bacteriostatic action of medicant adsorbed on carrier which has not been neutralized by subculture medium employed, each ring shall be transferred to a new tube of sterile medium and reincubated for an addnl period of 48 hrs at 37°. Results showing no growth on all 10 carriers would confirm the phenol coefficient number found. Results showing growth on any of the 10 carriers should be considered as indicating the phenol coefficient number to be an unsafe guide to the diln for use. In the latter case, the test should be repeated using lower dilns of the germicide under study. The maximum diln of the germicide which kills the test organism on the 10 carriers in the 10 min. interval would represent the maximum safe use-diln.

METHOD II

(Using *Micrococcus pyogenes* var. *aureus*)

Proceed as directed in Method I except to change phenol dilns and test organism. Use culture of *M. pyogenes* var. *aureus* F.D.A. 209, A.T.C.C. No. 6538 having at

least the resistance specified for the 24 hr culture at 20° in the phenol coefficient method.

If the germicide does not kill the test organism on 10 of the 10 carriers at the dilution indicated to be safe by the *S. typhosa* coefficient found and/or claimed, then it should not be recommended at this dilution for disinfecting in hospitals or places where pyogenic bacteria are likely to have special significance.

Results secured on selected samples of commercial germicides of different types when using the methods outlined are presented in Table 1.

In selecting the germicides listed in Table 1, primary consideration was given to securing examples of the variety of results which may be expected with the different types. The ones selected should not be considered as representative of all available germicides of the types listed.

With the 5 pine oil disinfectants having phenol coefficients ranging from 3.0 to 5.0, the maximum safe use-dilutions indicated by the coefficients claimed were confirmed by the use-dilution test when *S. cholerasuis* was employed. When the use-dilution test was employed using *M. pyogenes* var. *aureus*, two of these killed the organism at a dilution of 1:5, and the other three killed the organism when applied undiluted. While these results indicate that this class of products would not be effective against pyogenic bacteria when used at any practical dilution, they were completely out of line with previous experiences with pine oil disinfectants. In the past, products of this class have not been found effective against pyogenic bacteria at any dilution. Further study showed that these products also killed *M. pyogenes* var. *aureus* when used undiluted or at a dilution of 1:5 in the phenol coefficient procedure, although not at higher dilutions. This characteristic was apparently due to the use of synthetic anionic detergents in the emulsifiers employed which were germicidal at low dilutions for gram positive organisms. This activity does not seem to be great enough to have any practical significance, but seems to be characteristic of many pine oil disinfectants currently being produced. This effect will be investigated further.

With the 5 pine odor germicides, the results are considerably more variable. This might be expected from the more heterogenic character of the formulas employed with this class of materials. With germicide 1, a phenol coefficient of 5.0 was claimed and found. However, the highest dilution found to be effective in the use-dilution confirmation tests was 1:5. This would seem to indicate that the phenol coefficient claim of 5.0 was misleading. With germicide 2, a phenol coefficient of 3.0 was claimed and found. The maximum safe use-dilution of 1:60 indicated by this value was confirmed in the use-dilution test using *S. cholerasuis*. However, it was not effective against pyogenic bacteria even when tested undiluted in the use-dilution test using *M. pyogenes* var. *aureus*. A similar

TABLE 1.—*Results obtained with selected commercial germicides of different types in the phenol coefficient and use-dilution confirmation tests*

GERMICIDES BY TYPE	PHENOL COEFFICIENT (*S. typhosa* at 20°C.)		USE-DILUTION CONFIRMATION TESTS*	
	CLAIMED	FOUND	MAX. SAFE USE DILUTION (*S. cholerasuis*)	MAX. SAFE USE DILUTION (*M. pyogenes var. aureus*)
(Pine Oil)				
1	5.0	5.0	1:100	Undiluted
2	3.0	3.0	1:60	1:5
3	5.0	5.1	1:100	Undiluted
4	5.0	4.7	1:100	1:5
5	4.0	4.2	1:80	Undiluted
(Pine Odor)				
1	5.0	5.0	1:5	1:5
2	3.0	3.0	1:60	None
3	5.0	5.0	1:100	1:5
4	5.0	5.0	1:100	None
5	5.0	5.0	1:20	1:100
(Phenol Emulsifying Type)				
1	5.0	5.2	1:40	1:20
2	5.0	5.0	1:100	1:20
3	5.0	4.7	1:80	1:20
4	5.0	5.2	1:60	1:5
5	5.0	5.0	1:40	1:80
6	32.0	36.0	1:640	1:640
(Phenolic Soluble Type)				
1	4.0	4.0	1:80	1:40
2	4.0	4.2	1:80	1:80
3	5.0	6.7	1:100	1:80
4	4.0	4.4	1:80	1:80
(Quaternary Ammonium)				
1	25.0	25.0	1:100	1:100
2	20.0	22.2	1:50	1:50
3	25.0	23.3	None	None
4	5.0	5.0	1:5	1:5
5	25.0	25.6	1:60	1:60

* The maximum dilutions tested were no greater than those indicated to be safe by conventional calculations using the phenol coefficients claimed.

result was secured with germicide 4 of this series where a phenol coefficient of 5.0 was claimed and found. With germicide 3, where a phenol coefficient of 5.0 was also claimed and found, confirmation was secured in the use-dilution test with *S. cholerasuis,* but the maximum safe use-dilution with *M. pyogenes* var. *aureus* was only 1:5. Germicide 5 of this group possessed the phenol coefficient of 5.0 claimed for *S. typhosa,* but in the use-dilution test, a 1:20 dilution was the maximum which would kill *S. cholerasuis*

on 10 out of 10 carriers. On the other hand, this product was effective in the use-dilution test, at the dilution indicated to be safe, when *M. pyogenes* var. *aureus* was employed. This result is the reverse of what might normally be expected, but seems to have been due to the presence of phenols specific for Gram positive bacteria. With the 6 phenolic disinfectants of the emulsifying type, only samples 2 and 6 gave results in the use-dilution test with *S. cholerasuis* which confirmed the phenol coefficient claims. With sample 1, a phenol coefficient of 5.0 was claimed and a value of 5.2 was found. However, a dilution of 1:40 was necessary to kill *S. cholerasuis* in the confirmation test. With samples 3, 4, and 5, coefficients of 5.0 were also claimed against *S. typhosa*. Values of 4.7, 5.2, and 5.0 were found, respectively. Nevertheless, none of the three would kill *S. cholerasuis* in the use-dilution method at the indicated safe use-dilution of 1:100; dilutions of 1:80, 1:60, and 1:40 were necessary to secure this result. Sample 6 contained a high percentage of high boiling cresols, but was essentially an emulsion type product. Only one of the 6 products in this class killed *M. pyogenes* var. *aureus* in the use-dilution test at the dilution indicated to be safe by the *S. typhosa* coefficient. With sample 5, *M. pyogenes* var. *aureus* was killed at a higher dilution than *S. cholerasuis*. This was apparently due to the presence of synthetic phenols specific for Gram positive organisms. With sample 1, a use-solution twice as concentrated as that necessary with *S. cholerasuis* was required when *M. pyogenes* var. *aureus* was used. With sample 2, a use-solution 5 times as concentrated was necessary; with sample 3, a use-solution 4 times as concentrated was required, and with sample 4, a solution 12 times as concentrated was necessary. The failure to kill *M. pyogenes* var. *aureus* at the dilutions indicated to be safe by the *S. typhosa* coefficient, or the use-dilution test using *S. cholerasuis*, cannot be considered as unusual in the light of the reports of Brewer and Ruehle (5) and Klarmann and Shternov (6) which point out the weakness of the *S. typhosa* coefficient for determining the effectiveness of products of this class as disinfectants for pyogenic bacteria.

With each of the 4 phenolic disinfectants of the soluble type, *S. cholerasuis* was killed in the use-dilution test at the dilution indicated to be safe by the phenol coefficient claimed for *S. typhosa*. With samples 2 and 4, this dilution was also found to be effective when *M. pyogenes* var. *aureus* was employed in the confirmation test. With samples 1 and 3, higher concentrations were necesssary to kill *M. pyogenes* var. *aureus* than were required for *S. cholerasuis*.

No germicides of the quaternary ammonium type have been found which will kill either *S. cholerasuis* or *M. pyogenes* var. *aureus* in the confirmation tests at the dilutions indicated to be safe by the *S. typhosa* coefficients claimed and found. With germicide 1, a dilution of 1:100 was found to be necessary to kill both organisms in the confirmation methods, although a dilution of 1:500 was indicated to be safe by the phenol co-

efficient claimed and found. Similarly germicide 2 was found to require a 1:50 dilution to kill both organisms in the confirmatory methods, although a dilution of 1:400 was indicated by the *S. typhosa* coefficient claimed and found. With germicide 3, tests showed that the product would not disinfect in the confirmatory method with either organism, even when used undiluted, although definite phenol coefficients values could be secured. The results with germicides 4 and 5 were similar to those found with germicides 1 and 2 of this group.

TABLE 2.—*Comparison of results on common phenolic germicides by the phenol coefficient test and the use-dilution confirmation test. Test organism*—M. pyogenes *var.* aureus

GERMICIDE	PHENOL COEFFICIENT FOUND	HIGHEST 10 MIN. KILLING DILN. IN PHENOL COEFF. METHOD	HIGHEST 10 MIN. KILLING DILN. IN USE-DILN. CONFIRMATION METHOD	POSSIBLE SAFE USE-DILN.*
1	3.7	1:220	1:80	1:74
2	3.2	1:200	1:80	1:64
3	3.0	1:180	1:80	1:60
4	3.0	1:180	1:80	1:60
5	2.5	1:150	1:60	1:50
6	1.6	1:110	1:40	1:32
7	1.0	1:60	1:30	1:20
8	0.3	1:18	1:5	1:6

* Determined by multiplying phenol coeff. number by 20.

In Table 2, results by the phenol coefficient method on selected phenolic disinfectants of both the emulsifiable and soluble types are compared with those by the use-dilution confirmation method. *M. pyogenes* var. *aureus* was used as the test organism.

The data in Table 2 show clearly that a much lower dilution is required to disinfect carriers contaminated with *M. pyogenes* var. *aureus* in the use-dilution confirmation test than is required to kill this test organism in the phenol coefficient procedure. However, the critical killing dilution in the method does appear to be slightly higher than a theoretically safe use-dilution determined by multiplying the *M. pyogenes* var. *aureus* coefficient found by the number 20 to determine the number of parts of water in which one part of germicide should be incorporated.

While a variety of actual use tests have been conducted to determine the relative efficacies of the phenol coefficient and use-dilution confirmation tests as indices to practical disinfecting values, only one test conducted on floors and one test conducted on surgical instruments will be reported herein by way of illustration.

In the use tests on floors, the following procedure was employed: One hundred grams of chicken feces were mascerated in 100 ml of a 48 hr broth culture of *S.*

cholerasuis and then added with vigorous stirring to 10 quarts of water in an enamel pail. This water was then employed to mop up a ceramic tile floor in a room of approximately 10 by 15 feet. After drying, the floor was visually clean of excess organic matter, but was at the same time heavily contaminated with bacteria and soluble organic matter. The individual tiles in the floor were approximately 6 inches square. Using sterile cotton swabs, individual tiles were thoroughly wet with various dilutions of the selected germicides. At the conclusion of a 10 min. interval, each treated tile and untreated control tiles were swabbed with standardized sterile dry cotton swabs, and these were immediately taken into the laboratory for bacteriological analysis. This consisted of transferring the cotton swab into a 100 ml. sterile dilution blank containing a 5% aqueous solution of Tamol N and shaking thoroughly. From this initial suspension, dilution plate counts using nutrient agar were made. Also, 1 ml aliquots were used to inoculate tubes of lactose broth (which were incubated for presumptive evidence of the presence of coliform bacteria) and Bacto tetrathionate broth to recover surviving cells of *Salmonella*. All agar plates were incubated at 37°C. for 48 hrs. and counts were made using a Quebec colony counter. The lactose broth tubes were incubated at 37°C. for 48 hrs, after which time the growth in all tubes showing gas production was streaked on E.M.B. agar plates for incubation and identification of *E. coli* colonies. The tetrathionate broth tubes were incubated for 24 hrs. at 37°C., and then streaked out on Bacto SS agar plates for the isolation of colonies of *Salmonella*.

The results secured in this study are given in Table 3.

The data in Table 3 clearly indicate that the maximum safe use-dilution found in the use-dilution confirmation test using *S. cholerasuis* will provide for the disinfection of floors even when the maximum safe use-dilution indicated by the conventional method of interpreting the phenol coefficient found does not. With phenolic disinfectant 1, a phenol coefficient of 4.0 was claimed and found. This indicated that the maximum safe use-dilution to disinfect would be 1:80. This dilution was found to be adequate in the use-dilution confirmation test using *S. cholerasuis*. When tested on the floor, it reduced the total bacterial count of the floor by 99.918 per cent and eliminated the coliform and *Salmonella* organisms known to be present. On the other hand, phenolic disinfectants 2 and 3 which had phenol coefficients of 5.0 did not kill at the expected safe use-dilution of 1:100 in the use-dilution confirmation test using *S. cholerasuis*. They did kill at dilutions of 1:40 and 1:60, respectively, in this test. When tested on the floor at a dilution of 1:100, neither product eliminated the coliform and *Salmonella* organisms known to be present, although they did reduce the total bacterial counts by 93.299 and 98.609 per cent, respectively. At the dilutions of 1:40 and 1:60 indicated to be safe by the use-dilution confirmation test using *S. cholerasuis*, the total bacterial counts were reduced by 99.755 and 99.745 per cent and all coliform and *Salmonella* organisms were killed. With the Pine-Odor Quaternary Ammonium preparation, a safe use-dilution of 1:100 was indicated by the phenol coefficient claimed and found. This product did not kill in the use-dilution confirmation test using *S. cholerasuis* at a dilution of 1:100, but did kill in this test as a dilution of 1:5. When tested on the floor at

TABLE 3.—*Correlation of results obtained in phenol coefficient and use-dilution confirmation methods with results of floor disinfecting studies*

GERMICIDE	MAXIMUM SAFE USE-DILN. INDICATED BY:		DILUTIONS TESTED ON FLOOR	NUTRIENT AGAR PLATE COUNTS		BACTERIOLOGICAL CONFIRMATION TESTS FOR	
	S. typhosa COEFFICIENT	CONFIRMATION TEST USING *S. cholerasius*		AVERAGE COUNT PER 6" SQUARE TILE SWAB	PER CENT REDUCTION OVER CONTROL	*Coli*	*Salmonella*
Untreated control	—	—	—	98,599,000	0	+	+
Phenolic disinfectant 1	1–80	1–80	1–80	80,000	99.918	−	−
Phenolic disinfectant 2	1–100	1–40	1–100 1–40	5,600,000 140,000	93.299 99.755	+ −	+ −
Phenolic disinfectant 3	1–100	1–60	1–100 1–60	1,370,000 150,000	98.609 99.745	+ −	+ −
Pine Odor quaternary ammonium prepn	1–100	1–5	1–100 1–5	560,000 10,000	99.431 99.989	+ −	+ −
Pine Oil disinfectant	1–100	1–100	1–100	140,000	99.755	−	−
Phenol	1–20	—	1–20	20,000	99.979	−	−

dilutions of 1:100 and 1:5, the total bacterial counts were reduced by 99.431 and 99.989 per cent respectively, but at the 1:100 dilution the coliform and *Salmonella* organisms known to be present were not eliminated. They were eliminated at the 1:5 dilution. The pine oil disinfectant which was found to have a phenol coefficient of 5.0 killed *S. cholerasuis* at a dilution of 1:100 in the use-dilution confirmation method. When tested on the floor it reduced the bacterial count by 99.755 per cent and eliminated both the coliform bacteria and *Salmonella*. A 1:20 dilution of phenol which was tested on the floor as a control reduced the total bacterial count by 99.979 per cent and eliminated all coliform bacteria and *Salmonella*.

In the study on surgical instruments, 5 typical pyogenic bacteria were used; namely, *Streptococcus pyogenes*, *Streptococcus fecalis*, *Streptococcus agalaeticae*, *Micrococcus pyogenes* var. *albus*, and *Micrococcus pyogenes* var. *aureus*. These were grown in a 50–50 mixture of soy broth and whole blood. Twenty-four hour cultures, incubated in this medium at 37.5°C., were employed for contaminating heat sterilized detachable scalpel blades. All blades were drained and dried for 10 minutes before exposure for 10 minutes at 20°C. to selected dilutions of a phenolic disinfectant of

the emulsifiable type. It had been determined that the phenolic germicide used in this study possessed, according to the *S. typhosa* phenol coefficient claimed and found, a possible safe use-dilution of 1:100; a maximum killing dilution against *S. cholerasuis* in the use-dilution confirmation test of 1:60; a maximum killing dilution against *M. pyogenes* var. *aureus* in the phenol coefficient method of 1:20; and a maximum killing dilution against *M. pyogenes* var. *aureus* in the use-dilution confirmation test of 1:5. These four dilutions were employed in the study.

Ten contaminated blades were exposed to each dilution for 10 minutes with each test organism. Subcultures were made in soy broth with 0.1 per cent added whole blood and were incubated for 48 hours at 37°C. All blades exposed to the 1:5 dilution were subcultured in fresh tubes of media and incubated for an additional period of 48 hours, since sufficient medicant was carried over in the first transfer to cause the formation of cloudy precipitates in the first subculture tubes.

The results are reported in Table 4.

TABLE 4.—*Results of tests on a phenolic disinfectant of the emulsifiable type using detachable scalpel blades contaminated with a blood film carrying various pyogenic bacteria*

	POSSIBLE SAFE USE-DILUTION TESTED AS INDICATED BY:							
	S. typhosa COEFF.		*S. cholerasius* CONFIRMATION TEST		*M. pyogenes* VAR. *aureus* 10 MIN. KILLING DILN. COEFF. PROCEDURE		*M. pyogenes* VAR. *aureus* CONFIRMATION TEST	
DILUTION	1:100		1:60		1:20		1:5	
ORGANISM	NUMBER BLADES TESTED	NUMBER BLADES +*	NUMBER BLADES TESTED	NUMBER BLADES +	NUMBER BLADES TESTED	NUMBER BLADES +	NUMBER BLADES TESTED	NUMBER BLADES +
Strep. pyogenes	10	10	10	10	10	0	10	0
Strep. fecalis	10	10	10	9	10	0	10	0
Strep. agalacticae	10	10	10	2	10	0	10	0
M. pyogenes var. *albus*	10	10	10	10	10	10	10	0
M. pyogenes var. *aureus*	10	10	10	10	10	10	10	0

* Indicates growth in subculture medium.

The data in Table 4 show clearly that the dilution of 1:100 indicated to be safe by the *S. typhosa* coefficient claimed and found would not disinfect surgical blades contaminated with a blood film in the presence of any of the five pyogenic organisms used. The dilution of 1:60 found to be effective against *S. cholerasuis* in the use-dilution confirmation test was also ineffective against all five pyogenic bacteria. The effective killing dilution of 1:20 found in the phenol coefficient procedure using *M. pyogenes* var. *aureus* was effective in disinfecting the blades when the three streptococci were employed. It was not effective in the case of the two staphytococci. All blades were disinfected at the dilution of 1:5 indicated to be safe in the use-dilution confirmation test with *M. pyogenes* var. *aureus*.

The procedures outlined were checked for precision in collaborative investigations in which two Federal laboratories, one State laboratory, two commercial testing laboratories, and two manufacturers' laboratories participated. Two unknown phenolic type disinfectants were employed. Sample 1 was of the soluble type and sample 2 of the emulsifiable type.

The results have been summarized in Table 5.

Table 5 shows excellent agreement between the results of collaborators 1, 2, 3, 4, 5, and 6. Collaborator 7 found no end point with either germicide in any of the tests. The reason for this is not clear, but it would seem to be the inability to maintain the test cultures at the desired resistance levels to phenol. (The culture of *Salmonella cholerasuis* used was more resistant than specified in the procedure outlined and the culture of *M. pyogenes* var. *aureus* considerably less resistant than prescribed.)

A phenol coefficient of 4.0 had been claimed and found for unknown germicide 1. All seven laboratories found that this product killed *Salmonella cholerasuis* in the use-dilution confirmation test at the dilution of 1:80 indicated to be safe by this value (4 times 20). Six of the seven laboratories found that this product would also kill *M. pyogenes* var. *aureus* at a dilution of 1:80 in the use-dilution confirmation test. One found that a 1:60 dilution was necessary to secure this result.

With germicide 2, a phenol coefficient of 5.0 was claimed and found. Only 2 of the 7 collaborators found that the indicated safe use-dilution of 1:100 would kill *Salmonella cholerasuis* in the use-dilution confirmation test. The other 5 laboratories agreed that a 1:60 dilution was necessary to secure this result. Only one laboratory found that the dilution of 1:100 would kill *M. pyogenes* var. *aureus* in the use-dilution confirmation method. One found that a dilution of 1:10 was necessary for disinfection in this method, 3 found that a dilution of 1:5 was required, and 2 found that disinfection was not secured even at a dilution of 1:5.

DISCUSSION

These studies show that the described use-dilution confirmation tests can be applied, along with the existing phenol coefficient procedures, to provide a more accurate index than phenol coefficients alone to the actual value of chemical germicides for disinfecting articles, surfaces, and places where prior cleaning cannot be depended upon to remove all interfering organic matter or to reduce bacterial loads to low levels. The collaborative data reported clearly indicate that the procedures have sufficient precision for use in referee work.

The employment of multiple ring carriers at each dilution to be tested makes the procedures too cumbersome for most initial evaluations. However, ten carriers are necessary for the final determination of 100 per cent kill end points with many of the newer types of germicides which are active in very high dilution. The single and admittedly arbitrary time

TABLE 5.—*Results of collaborative studies on the use-dilution confirmation methods with two unknown phenolic disinfectants*

UNKNOWN PHENOLIC GERMICIDES	DILUTION	COLLABORATING LABORATORIES													
		1		2		3		4		5		6		7	
		NUMBER RINGS TESTED	NUMBER RINGS +	NUMBER RINGS TESTED	NUMBER RINGS +	NUMBER RINGS TESTED	NUMBER RINGS +	NUMBER RINGS TESTED	NUMBER RINGS +	NUMBER RINGS TESTED	NUMBER RINGS +	NUMBER RINGS TESTED	NUMBER RINGS +	NUMBER RINGS TESTED	NUMBER RINGS +
(Method I. Using *Salmonella cholerasuis*)															
1 (Soluble type)	1:80	9	0	10	0	10	0	10	0	10	0[1]	10	0	10	0
	1:60	10	0	10	0	10	0	10	0	10	0	10	0	10	0
2 (Emulsion type)	1:100	10	9	10	6	10	3	10	4	10	0[4]	10	4	10	0
	1:80	10	3	10	3	10	2	10	1	—	—	10	1	10	0
	1:60	10	0	10	0	10	0	10	0	—	—	10	0	10	0
Phenol Resistance Test Cultures		1:90		1:90		1:95		1:80		1:85		1:90		1:90	
(Method II. Using *M. pyogenes* var. *aureus*)															
1 (Soluble type)	1:80	9	0	10	5	10	0	10	0	10	0[3]	10	0	10	0
	1:60	10	0	10	0	10	0	10	0	10	0	10	0	10	0
2 (Emulsion type)	1:100	10	10	8	8	10	10	10	10	—	—	—	—	10	0
	1:80	10	10	—	—	10	10	10	10	—	—	—	—	—	—
	1:60	10	10	8	8	10	10	10	10	—	—	10	10	10	0
	1:20	10	9	10	10	10	9	10	4	10	10	10	4	10	0
	1:10	—	—	—	—	—	—	10	0	—	—	—	—	—	—
	1:5	10	0	10	10[2]	10	0	—	—	10	3	10	0	10	0
Phenol Resistance Test Cultures		1:60		1:60		1:60		1:60		1:65		1:65		1:80	

[1] Collaborator 5 reported no + rings at dilutions indicated and some − rings at all dilutions up to 1–500.
[2] Collaborator 2 reported that some negative results at dilution of 1:20 and 1:5 were found but were traced by subculture to the bacteriostatic effect of small amounts of the germicide carried into the subculture tubes.
[3] Collaborator 5 reported no + rings at a dilution of 1–100 but 9 + rings at 1:120.
[4] Collaborator 5 reported 6 + rings at a dilution of 1:120.

interval of 10 minutes is longer than the contact period which would be encountered in some applications, but represents about the average time that activity could be expected on floors, walls, and fixed equipment after mopping or spraying, since drying is usually quite complete within this time interval.

From the illustrations given it is apparent that, with some products, use of the phenol coefficient values according to the conventional method of calculating safe use-dilutions may not provide solutions which can be depended upon to disinfect. Where this is true, the phenol coefficient number can be misleading in that it provides an erroneous index to the true disinfecting value of the product. Consideration should be given, therefore, to prohibiting the use in labeling of coefficient numbers higher than those that can be confirmed by the use-dilution confirmation tests described.

SUMMARY

Two use-dilution test procedures for disinfectants have been described. One employs *Salmonella cholerasuis* as the test organism, the other *Micrococcus pyogenes* var. *aureus*.

Results secured by these procedures have been compared with results secured in the official phenol coefficient methods and in actual use tests on floors and surgical instruments. These comparisons indicate clearly that the use-dilution procedures described can be applied in supplementing phenol coefficient data to provide more accurate evaluations as to the safe use-dilutions of chemical germicides.

Both of these use-dilution tests have been evaluated collaboratively by seven laboratories and the results reported have been analyzed. These results indicate that the tests have sufficient precision to warrant use for referee purposes.

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THE RESISTANCE OF BACTERIAL SPORES TO CONSTANT BOILING HYDROCHLORIC ACID*

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Great variation is known to exist in the resistance of the endospores found in strains of individual *Bacilli* and *Clostridia*. For a proper evaluation of chemicals represented as sporocidal it is therefore essential to employ test culture spores carrying some predetermined resistance to a known chemical. Hydrochloric acid has been shown to be sporocidal and it has been the policy of the Insecticide Division to use this chemical as a standard for determining the resistance of spores used in tests of the sporocidal activity of commercial germicides.

Possibly the most common method for the standardization of hydrochloric acid is the constant boiling method. The procedure is outlined in most textbooks on quantitative analysis such as Kolthoff and Sandell (1) and in the *Official Methods of Analysis* (2). Thus, constant boiling hydrochloric acid can be considered as both a convenient and commonly recognized chemical standard. The exact hydrochloric acid concentration may vary slightly, depending on the atmospheric pressure. At 780 mm Hg the concentration will be 20.173 per cent and at 730 mm it will be 20.293 per cent or a mean molarity of approximately 5.5. The acid prepared and used in these studies had a concentration of 20.210 per cent.

In private communications, some bacteriologists have claimed that they could not obtain bacterial spores which would resist constant boiling hydrochloric acid solutions for measurable periods of time at 20°C. Also, it has been observed in the laboratory of this Division that many stock cultures of *Bacilli* and *Clostridia* do not produce spores that will withstand boiling hydrochloric acid at 20°C. On the other hand, it has been found that cultures of almost any species of these two genera can eventually be induced to produce spores which, when dried, will withstand this solution for 5 minutes. Many species can be induced to produce spores which will withstand this treatment for 30 minutes or longer. The various procedures employed in the Division's laboratories in obtaining resistant spores for testing purposes, exposing them to the hydrochloric acid standard as well as the unknown germicides, and subculturing to determine death of the spores, have been carefully evaluated and a preferred

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