

BACTERIOLOGICAL CULTURE OF WATER-IN-OIL EMULSIONS

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ADMINISTRATION to man of vaccines and allergenic extracts emulsified in mineral oil has been increasing in the past 10 years.^{1, 2, 3} Studies have shown that aqueous extracts of substances such as pollens, house dust, and insect venom can be effectively given to patients with allergy in one or several injections as a water-in-oil emulsion. Results of such treatment are, in most reports, at least as effective as older perennial or preseasonal multiple-injection programs.²⁻⁵ All allergenic extracts must be bacteriologically sterile before they can be safely used. The usual technique for culturing aqueous extracts is direct addition to thioglycollate broth and incubation at 37° C. for 48 hours,^{6, 7, 8} which will detect both aerobic and anaerobic organisms. This same technique is used for culturing emulsions to be used in injections in humans.⁹

This experiment was planned to determine if the usual cultural methods would be sufficient to verify emulsion sterility, and, if not, what techniques would be necessary to detect bacterial contamination. Data will be presented to show that emulsions contaminated with three common organisms (*staphylococcus*, *streptococcus*, and *Clostridium welchii*) must be broken into small particles prior to addition to culture media to reliably detect bacterial contamination. It will also be shown that stored emulsions tend to become less contaminated with passage of time. Finally, it will be shown that bacteria do escape from emulsions administered to mice.

MATERIALS AND METHODS

Preparation and Contamination of Emulsions.—Water-in-oil emulsions for culture were prepared by using equal parts of Daroil† and buffered aqueous diluent‡ (NaCl, 0.5 per cent; KH₂PO₄, 0.33 per cent; Na₂HPO₄ 12H₂O, 0.14 per cent; phenol, 0.4 per cent). One milliliter of a one to one million dilution of a 4-hour culture of Group A Type 17 streptococcus, *Staphylococcus albus* (coagulase and mannitol +), or *Clostridium welchii* was added to 4 ml. of diluent just prior to preparation of the emulsions. Approximately ten organisms were

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calculated to be present in each milliliter of emulsion on the basis of plate counts on dilutions of the same 4-hour cultures.^{6, 7, 8} After specific contamination, emulsification was achieved by passing both the aqueous and oil phases through a double hub No. 15 needle and Swinney adapter* forty times, with the use of a sterile technique. Stability of the emulsions was checked by physical appearance, microscopic appearance, and drop on water test. An emulsion containing Group A Type 17 streptococcus for administration to mice was similarly prepared, with the substitution of Difeo Todd-Hewitt broth for the water phase.

Bacteriologic Culture of Emulsions.—Each of the three specifically contaminated emulsions were cultured in quadruplicate by adding one milliliter to each of four tubes of Todd-Hewitt broth and to four tubes of thioglycollate broth and then by incubating them at 37° C. for one week in normal atmospheric conditions. In addition, one milliliter of each contaminated emulsion was suspended in 4 ml. of Todd-Hewitt broth by again passing both through a sterile double hub No. 15 needle and Swinney adapter fifteen times (hereafter called suspended emulsion). Suspended emulsions were then cultured immediately by adding one milliliter (0.20 ml. of original emulsion) to four tubes of each of the two kinds of broth and incubating them at 37° C. for one week at normal atmospheric conditions.

Effect of Storage Time and Temperature on Contaminated Emulsions.—Different sets of contaminated emulsions were stored at 4° C., at 37° C., and at room temperature for a period of 3 weeks. At the end of each week, suspended emulsions were prepared from them and cultured as previously described.

Administration of Contaminated Emulsion to Mice.—Ten white mice were injected intraperitoneally and 10 were injected subcutaneously with 0.5 ml. of contaminated emulsion each (approximately 5 organisms per mouse). Equal numbers of mice received the same number of organisms in Todd-Hewitt broth by the same routes. Survival time for each mouse was recorded. Mouse blood was cultured post mortem and isolated organisms were identified by the serologic methods of Lancefield.¹⁰ A contaminated emulsion was placed in storage at 37° C. for a period of 3 weeks. At the end of this storage period it was injected intraperitoneally into 10 white mice.

Identification of Positive Cultures.—All cultures suspected of being positive by gross appearance at the end of one week were inoculated into Todd-Hewitt broth, thioglycollate broth, and streaked on blood agar. In cases in which subculture growth was positive, growth characteristics were noted and a morphological identification was made on a Gram stain. If growth could not be recognized by these methods the culture was reported as *No Growth*.

RESULTS

Emulsions contaminated with streptococcus, staphylococcus, and *Clostridium welchii*, which were added immediately to two types of culture media and incubated for one week at 37° C., gave variable results. Two of eight cultures

*Milipore Filter Corp., Bedford, Massachusetts.

TABLE I. COMPARISON OF THE DIRECT AND SUSPENDED METHODS OF CULTURING CONTAMINATED EMULSIONS

ORGANISMS IN EMULSION	METHOD OF CULTURE	NUMBER OF POSITIVE	NUMBER OF CULTURES
		THIOGLYCOLLATE BROTH	TODD-HEWITT BROTH
Streptococcus Group A Type 17	Direct	3/4	1/4
	Suspended emulsion	4/4	4/4
Staphylococcus mannitol + coagulase +	Direct	3/4	1/4
	Suspended emulsion	4/4	4/4
<i>Clostridium welchii</i>	Direct	3/4	—
	Suspended emulsion	4/4	—
Control (No bacteria)	Direct	0/4	0/4
Control (No bacteria)	Suspended emulsion	0/4	0/4

were positive in Todd-Hewitt broth and 9 out of 12 in thioglycollate tubes (Table I). When the same contaminated emulsions were first mechanically suspended in Todd-Hewitt broth, growth was apparent in all tubes, although only one fifth of the number of organisms were added from the suspended emulsions as compared to the direct culture of the original emulsions. Uncontaminated sterile emulsions remained sterile throughout the study.

The effect of temperature and time of storage was tested with the use of the suspended emulsion technique (Table II). Although cultures were uniformly positive when inoculated immediately, only the staphylococcus cultures were positive after 2 weeks storage and, with one exception, these were positive only in those emulsions stored at 37° C. After 3 weeks of storage, only four of eight tubes from the staphylococcus emulsions stored at 37° C. were positive. All

TABLE II. EFFECT OF TIME AND TEMPERATURE OF STORAGE ON SURVIVAL OF ORGANISMS IN CONTAMINATED CULTURES

ORGANISM IN EMULSION	STORAGE TEMP.	AFTER 1 WEEK OF STORAGE		AFTER 2 WEEKS OF STORAGE		AFTER 3 WEEKS OF STORAGE	
		THIO-GLYCOL-LATE	TODD-HEWITT	THIO-GLYCOL-LATE	TODD-HEWITT	THIO-GLYCOL-LATE	TODD-HEWITT
Streptococcus Group A Type 17	4° C.	4/4	1/4	0/4	0/4	0/4	0/4
	37° C.	4/4	2/4	0/4	0/4	0/4	0/4
	Room temp.	4/4	3/4	0/4	0/4	0/4	0/4
Staphylococcus mannitol + coagulase +	4° C.	4/4	4/4	1/4	0/4	0/4	0/4
	37° C.	4/4	4/4	4/4	4/4	2/4	2/4
	Room temp.	4/4	4/4	0/4	0/4	0/4	0/4
<i>Clostridium welchii</i>	4° C.	4/4	—	0/4	—	0/4	—
	37° C.	4/4	—	0/4	—	0/4	—
	Room temp.	4/4	—	0/4	—	0/4	—
Control (No organism)	4° C.	0/4	0/4	0/4	0/4	0/4	0/4
	37° C.	0/4	0/4	0/4	0/4	0/4	0/4
	Room temp.	0/4	0/4	0/4	0/4	0/4	0/4

TABLE III. COMPARATIVE SURVIVAL TIME IN MICE INJECTED WITH STREPTOCOCCI IN EMULSIONS AND IN BROTH

ROUTE OF ADMINISTRATION	MATERIAL INJECTED	ORGANISMS PER INJECTION	NO. OF MICE	AVERAGE SURVIVAL TIME (HR.)
Subcutaneous	Emulsion	5	10	108
	Broth	5	10	130
Intraperitoneal	Emulsion	5	10	26.1
	Broth	5	10	26.3
Intraperitoneal	3-week old emulsion	5	10	All survived 10 days

other cultures were negative. Streptococcus emulsions, stored 3 weeks at 37° C. and then administered intraperitoneally into 10 mice, showed all mice to be alive at the end of 10 days (Table III).

Group A Type 17 streptococcus emulsified and injected immediately produced death in all mice (Table III). This organism was recovered from the blood of these mice at postmortem examination. Mice survived much longer after subcutaneous injection than after intraperitoneal injection. The average time of death after the intraperitoneal injection of organisms in broth was not significantly different from the time of death when the same number of organisms were given in emulsion (Table III).

COMMENT

The usual method of culturing allergenic extracts by the simple addition to culture media has been shown to be unsatisfactory for culturing water-in-oil emulsions known to contain small numbers of streptococci, staphylococci, or *Clostridium welchii*. A preliminary step of suspending the emulsion in Todd-Hewitt broth enhanced the possibility of obtaining growth. The success of such a method could be due to breaking of bacterial clumps or chains or to freeing the bacteria from the emulsion into the surrounding medium.

All the bacteria tested appear to decrease in number with the passage of time at the temperatures used. The water phase contains a small amount of medium and a small amount of phenol. These two factors could explain why death takes place in time. The possibility that the bacteria are not dead, only nonviable, on artificial media seems unlikely since a stored, streptococci-contaminated emulsion did not kill mice after 3 weeks. The possibility that mineral oil or Arlacel is toxic to bacteria was not tested. Spores are not usually present in new *Clostridium* cultures, therefore the survival of spores in emulsion was not tested. Even though the number of bacteria do tend to decrease with increasing storage time, one could not be confident that all bacteria would die and eventually produce a sterile emulsion.

Emulsions given subcutaneously to mice appear to remain fairly well localized. When given intraperitoneally they spread throughout the abdomen rather quickly. The differences in times of death noted from intraperitoneal and subcutaneous injections could be attributed to this mechanical dispersion difference.

Using either route, the bacteria eventually escape to cause the death of all the mice. When a similar emulsion is placed in proper culture media, frequently no growth will result. Perhaps the natural movements of the animal enable the bacteria to escape from the injected emulsion. The possibility of a surface active agent being present around the injected emulsion was not investigated but could explain the differences noted. It seems apparent that even a slightly contaminated emulsion would be likely to release bacteria after injection and possibly cause a local or systemic infection.

SUMMARY

1. Mechanical suspension of emulsions in broth prior to culturing is a more reliable way to detect contamination with small numbers of bacteria than the direct addition of the emulsion to thioglycollate broth.
2. Storage of emulsions tends to reduce bacteriological contamination with streptococci, staphylococci, and to *Clostridium welchii*.
3. Bacteria readily escape from emulsions injected in mice.

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