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Method for Microbiological Testing of Nonsterile Pharmaceuticals

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A method for testing nonsterile pharmaceutical preparations for their microbial content is described. As far as possible, only solid culture media were used to obtain quantitative results. Aqueous and water-soluble products were tested with membrane-filter techniques. Nonfilterable products were first emulsified or suspended and the homogenate was used for examination. In both procedures, the total number of colonies is determined for aerobic bacteria and fungi. Tests for certain undesirable microbial groups were conducted with selected media. The method described is applicable for finished products, bulk products, raw materials, and active ingredients.

Microbiological testing of nonsterile pharmaceuticals has thus far been performed only in special cases. In recent years, however, it has been repeatedly shown that microbiological control of such products is necessary (1, 6, 7, 9, 10, 19). Until now, regulations concerning the permissible level of nonpathogenic microorganisms in nonsterile drugs exist in only a few countries (e.g., Czechoslovakia and Scandinavia). Proposed methods of examination have been made by Kallings et al. (7) and Dony and Gérard (6). However, these authors expressed different views about the permissible limits and method of examination. In formulating such regulations, consideration must be given to the promotion of hygiene and safety as well as to the feasibility of application in good manufacturing practice. To permit comparative testing of these products, uniform methods of examination are important. In 1966, several Swiss microbiologists entrusted with this task proposed a suitable technique of examination. The procedure recommended was based upon numerous preliminary tests on various drugs.

MATERIALS AND METHODS

Various considerations were decisive in choosing the method of examination. The method should be relatively simple, but enable users to obtain accurate and reproducibly quantitative results. As far as possible, generally known and universally obtainable culture media should be used. Selective media are used only for the demonstration of special groups of pathogens. The method should be as widely applicable as possible and suitable not only for finished pharmaceuticals but also for bulk products, raw materials, and active ingredients.

In view of these considerations and for technical reasons, we divided the pharmaceuticals into two categories: (i) aqueous and water-soluble products tested by the membrane filter method and incubation of the filters on solid media; and (ii) water-insoluble products homogenized in Tween 80 and phosphate buffer. Suitable homogeneous dilutions may be tested by the plate count technique and an enrichment method.

Precautionary measures against microbial contamination must be taken in the laboratory.

Media and general procedures. In general, the products are examined for aerobic bacteria, fungi, *Enterobacteriaceae*, and *Pseudomonas* species.

Determination of the total number of aerobic bacteria. The medium to be used for detecting aerobic bacteria is Trypticase Soy Agar (BBL). Both mesophilic and psychrophilic bacteria are detected by incubation at 30 C. A parallel test for anaerobes was initially made, but almost without exception the bacteria isolated were facultative anaerobes. Anaerobic cultivation is therefore of little practical significance and is done only in special cases.

Determination of the total number of fungi. The medium used for determination of fungi is Sabouraud Dextrose Agar incubated at 30 C. Various bacteria grow on this medium and, conversely, some molds and yeasts also grow on Trypticase Soy Agar. However, by repeated control of the cultures during the incubation period, it is possible to obtain a reliable evaluation.

Determination of Enterobacteriaceae. It is not the aim to detect only pathogenic representatives of this family of bacteria. *Enterobacteriaceae* in general are considered indicators of inadequate factory hy-

[1] Presented at the 68th Annual Meeting of The American Society for Microbiology, Detroit, Mich., 5-10 May 1968.

TABLE 1. *Filterable products*[a]

Type of organism to be detected	Amt of material filtered	Medium	Incubation temp	Time of inspection of cultures
	ml		*C*	
Aerobic bacteria.....	1	Trypticase Soy Agar	30	1, 2, and 5–7 days
(total count)	10		30	
Fungi.....	1	Sabouraud Dextrose Agar	30	2 and 5–7 days
(total count)	10		30	
Enterobacteriaceae....	10	Violet Red Bile Agar + 1% glucose	37	18–24 hr
Pseudomonas.....	1	Pseudosel agar	30	2 days
	10		30	

[a] Immediately after each filtration of material, the filters were washed out with 100 ml of a phosphate buffer solution containing 3.56 g of KH_2PO_4, 7.23 g of $Na_2HPO_4 \cdot H_2O$, 4.3 g of NaCl, 1.0 g of peptone, and 1,000 ml of distilled water (*p*H 7.0).

giene, provided the starting materials are uncontaminated (12). The membrane filter is incubated at 37 C on Violet Red Bile Agar (BBL) plus 1% glucose (17). Homogenized products are initially enriched in EE broth (BBL), the *Enterobacteriaceae* enrichment broth used by Mossell and others, (18, 20). For confirmation, subinoculation on violet red bile agar plus 1% glucose is used. On this medium, *Enterobacteriaceae* form purple-red to violet-red colonies. In the case of a positive finding, inoculation onto ordinary selective media can be performed and an accurate differentiation obtained by using biochemical or possibly serological testing and phage typing.

Determination of Pseudomonas species. Pseudomonas bacteria can cause difficulty in a nearly germ-free preparation (1, 7, 19). Owing to their resistance to many preservatives and their minimal nutrient requirements, these bacteria can often multiply in certain preparations. This test is therefore indicated for all susceptible products, or where there are other grounds for suspecting their presence. Pseudosel agar (BBL), incubated at 30 C, is used to detect *P. aeruginosa* as well as psychrophilic species (3). Colonies can be identified where necessary by using the oxidase test (8), and by other biochemical methods (4).

The standard examination outlined above can be expanded in certain cases for selective detection of other types of bacteria, e.g., detection of the following bacteria may be of practical significance: pathogenic staphylococci [detection with Vogel plus Johnson agar (BBL) or Chapman-Stone or Baird-Parker medium (Oxoid)]; enterococci [detection with M-UNH-enterococcus agar (BBL)]; *Clostridium perfringens* [detection with TSN agar (BBL), anaerobic incubation in a Brewer anaerobic jar or tubes as specified in the accompanying instructions]; *Bacillus cereus* [detection by the methods of Mossel and others (13–16)].

Testing of aqueous and water-soluble products. This category includes aqueous and water-soluble preparations for oral use, filterable raw materials, and active ingredients.

Equipment and filters needed are as follows: Millipore Hydrosol Filtration apparatus, Millipore Pyrex Filter Holder (Filter HAWG), or a similar apparatus manufactured by Sartorius Membranfilter GmbH (Filter MF 50). For preparations which attack the usual cellulose ester membranes, other inert filters of corresponding pore size may be used.

With finished products, the contents of three or more containers are mixed together per batch, producing at least 100 ml of liquid. This mixture is taken as the standard solution for subsequent tests. If bulk products, active ingredients, or raw materials are examined according to this method, the number of samples being adapted to the circumstances. Table 1 illustrates the methods employed in testing the samples.

Immediately after each filtration, the filters are washed with 100 ml of a solution having the following composition: KH_2PO_4, 3.56 g; $Na_2HPO_4 \cdot 2H_2O$, 7.23 g; NaCl, 4.3 g; peptone (e.g., Polypeptone, BBL, or Difco peptone), 1.0 g; distilled water to 1 liter (*p*H adjusted to 7.0).

Experiments of Straka and Stokes (21), Bowman (2), and Bühlmann (*unpublished results*) indicated that the addition of a small quantity of peptone to isotonic solutions aids in the survival and germination, especially in the case of damaged organisms. If there is difficulty in removing certain active substances from the filters, larger quantities of flushing liquid may be used. In solutions with mecurial preservatives, 0.05% sodium thioglycollate should be added to the medium. Immediately after rinsing is completed, the filters are laid on the recommended medium and incubated.

With filtration of 10 ml, the minimal number of detectable microorganisms is $\leqq 0.1$/ml for aqueous products, and $\leqq 1$/ml for dissolved products diluted 1:10.

Testing nonfilterable products. This category includes fatty or oily products, suspensions, water-insoluble solid pharmaceutical preparations, water-insoluble active ingredients, and raw materials.

TABLE 2. *Nonfilterable products*

Type of microorganism to be detected	Amt of homogenate inoculated	Medium	Incubation temp	Type of inoculation	Time of inspection of cultures	Minimal no. of microorganisms detectable in 1:10 dilution	Evaluation of results
	ml		*C*				
Aerobic bacteria (total count)	1	Trypticase Soy Agar	30	Incorporation in 20 ml of medium	1, 2, and 5–7 days	≷10/g	Avg of 2 parallel cultures
Fungi (total count)	1	Sabouraud Dextrose Agar	30	Incorporation in 20 ml of medium	2 and 5–7 days	≷10/g	Avg of 2 parallel cultures
Enterobacteriaceae	10	EE broth	37	Enrichment in 100 ml of EE broth for 18–24 hr, then subinoculation on Violet Red Bile Agar + 1% glucose	Confirmatory culture after 24 hr	≷1/g	With positive finding only approximate data possible
Pseudomonas	0.5	Pseudosel agar	30	Inoculation of surface	2 days	≷10/g	Addition of results of 2 parallel cultures

With finished products, samples must be collected from three or more containers and at least 10 g should be used for homogenization.

Homogenization of the material. Homogenization is carried out by the method proposed by Kallings et al. (7), in which 1 part of the material is well mixed with 1 part of sterile, warm Tween 80 and 8 parts of warm phosphate peptone-containing buffer solution previously mentioned in the filtration method.

To test fatty or oily products, Tween 80 and buffer solution are heated to 40 C for the homogenization. The test material should be at room temperature. It is possible either to mix the material with Tween 80 first and then add the buffer solution, or to mix Tween 80 and buffer solution first and then add the test material. Media are inoculated while the mixture is warm. If a stable emulsion cannot be obtained, the test is performed with the aqueous phase.

In tests of powder, tablets, coated tablets, and other materials, suspension of the test material may also be produced with Tween 80 and buffer solution or with the buffer solution alone. Products which cannot be suspended are thoroughly pulverized and, after most of the solid material has settled out, the supernatant fluid is used for inoculation. Suspensions or emulsions of the test material may be used without further processing for inoculation.

For preparation of the homogenate, sterile glass containers (screw-cap bottles, Erlenmeyer flasks) containing a sufficient number of glass beads (3 to 4 mm diameter) are usually used. If satisfactory homogenization is not attained in this way, an Ultra-Turrax (Janke and Kunkel) or similar device may be employed.

If the homogenate of the 1:10 dilution produces an excessively cloudy medium in the poured plates or contains growth-inhibiting substances which cannot be adequately inactivated, it is further diluted with buffer solution to 1:100. Where required, suitable inactivating agents, such as para-aminobenzoic acid with sulphonamides or serum, may be added to the medium. Table 2 shows details of the inoculation and evaluation.

CONCLUSIONS

It is justifiable to expect the absence of harmful organisms in nonsterile therapeutic products and that the level of other microorganisms does not exceed a fixed limit. The establishment of a stricter limit is indicated in nasal preparations and topical products for skin wounds (e.g.) than in other skin preparations and oral drug forms. The limits should be feasible with a reasonable technical outlay in the production plant and not cause an unnecessary increase in the price of the products.

Production of goods with a low microbial content can be achieved by strict industrial hygiene. In this connection, microbiological controls of air, water, working equipment, and personnel are significant (1, 6). A systematic control of the basic materials is also important. Above all, materials which are liable to be contaminated should be checked regularly and used only when they do not exceed the fixed limit of contamination.

DISCUSSION

Randomized samples must be selected in making all tests. Filtration of 1 and 10 ml or inoculation of the 1:10 homogenate has often proved satisfactory for the microbiological control of a product. With high contamination or for other reasons, it may be necessary to test gradually decreasing quantities of the material. In such cases, 1:10, 1:100, and 1:1,000 dilutions of the homogenized preparations are usually examined. Where there is difficulty in interpreting the significance of the bacterial counts, the same samples should be retested or, preferably, additional samples should be obtained.

When a product is tested for the first time, the possibility of carryover of the preservative agent should be checked by inoculating suitable test organisms on the medium inoculated with the test materials. Often colony counts on the higher dilutions must be relied upon for accurate counts if the inactivating agent is present in sufficient amount in the lower dilutions.

The purpose of these studies was not to determine sterility of the product but to determine the minimal detectable number of organisms present. If the viable count is below the determinable level, the results are recorded as $<10/g$ or $<0.1/ml$. Where the interpretation of the results is to be based on the types of microorganisms isolated and not the total count, details of the differential identification test should be given. In some water-insoluble products where only a very low level of contamination is acceptable, a qualitative method with direct inoculation into a liquid medium may be substituted for the described technique.

A preincubation of the test samples is important in foods and provisions in the control of preserved and semipreserved canned goods, especially to detect anaerobic gasogenic or toxin-forming microorganisms with greater assurance (5, 11). A similar preincubation was proposed by Dony and Gérard (6) for pharmaceutical products, but this appears pointless for pharmaceutical preparations for the following reasons. In food hygiene, the preincubation serves primarily to detect proteloytic, facultative, or strict anaerobes, whereas, in general, pharmaceutical preparations do not provide optimal conditions for anaerobic growth, and the materials used are usually a poor substrate for growth of proteolytic organisms.

It is of indisputable importance to know whether certain saprophytic or pathogenic organisms can grow in a preparation and whether an appropriate preservative is necessary. Such questions, however, must be answered in the development stage of the product by suitable spoilage or preservative tests (1).

In determining the frequency of investigations, it is advantageous to distinguish between two groups of preparations. (i) Products in which only a relatively small number of microorganisms can be tolerated or which are prnoe to contamination—in such cases, an examination of each batch of the product is indicated. (ii) Preparations with less rigid limits of contamination or which are less likely to be contaminated—such products can be controlled by periodic spot-checks.

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LITERATURE CITED

1. Bean, H. S. 1967. The microbiology of topical preparations in pharmaceutical practice. 2. Pharmaceutical aspects. Pharm. J. **199**:289–292.
2. Bowman, F. W. 1966. Application of membrane filtration to antibiotic quality control sterility testing. J. Pharm. Sci. **55**:818–821.
3. Bühlmann, X., H. Bruhin, und W. A. Vischer. 1961. Über einige Eigenschaften der psychrophilen Bakterien. Proc. Intern. Assoc. Vet. Food Hygienists, 2nd Symp. Basel. p. 217–220.
4. Bühlmann, X., W. A. Vischer, and H. Bruhin. 1961. Identification of apycyanogenic strains of *Pseudomonas aeruginosa*. J. Bacteriol. **82**: 787–788.
5. Buttiaux, R., and D. A. A. Mossel. 1957. L'analyse bactériologique des produits alimentaires périssables et conservés. Ann. Inst. Pasteur Lille **9**:138–175.
6. Dony, J., and P. Gérard. 1968. La contamination microbienne des médicaments et l'établissement de normes de qualité bactériologique. J. Mond. Pharm. **11**:19–32.
7. Kallings, L. O., F. Ernerfeldt, and L. Silverstolpe. 1966. Microbiological contamination of medical preparations. Report to the National Board of Health, 1965. Boktruckeri AB Thule, Stockholm.
8. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature **178**:703.
9. Lennington, K. R. 1967. Salmonella in drugs and dietary supplements. Drug Cosmetic Ind. **100**:42–43.
10. Lennington, K. R. 1967. Salmonella in drugs and dietary supplements. Drug Cosmetic Ind. **100**:174–175.
11. Mossel, D. A. A. 1956. Aufgaben und Durchführung der Modernen Hygienisch-Bakteriolo-

gischen Lebensmittelüberwachung. Wien. Tierärztl. Monatsschr. **43**:321–340 und 596–610.
12. Mossel, D. A. A. 1958. Die Verhütung der Verbreitung von Salmonellosen und Sonstigen Enterobacteriosen Durch Lebensmittel. Zentr. Bakteriol. Parasitenk. Abt. I. Ref. **166**:233–244.
13. Mossel, D. A. A. 1965. Die mikrobiologische Qualitätskontrolle der Margarine. Fette Seifen Anstrichmittel **67**:901–906.
14. Mossel, D. A. A. A. 1965. Die Mikrobiologische Qualitätskontrolle der Margarine. Fette Seifen Anstrichmittel **67**:1015–1018.
15. Mossel, D. A. A. A. 1966. Die mikrobiologische Qualitätskontrolle der Margarine. Fette Seifen Anstrichmittel **68**:487–491.
16. Mossel, D. A. A., M. J. Koopman, and E. Jongerius. 1966. The enumeration of *Bacillus cereus* in foods. Antonie van Leeuwenhoek, J. Microbiol. Serol. **32**:453.
17. Mossel, D. A. A., W. H. J. Mengerink, and H. H. Scholts. 1962. Use of a modified MacConkey agar medium for the selective growth and enumeration of *Enterobacteriaceae*. J. Bacteriol. **84**:381.
18. Mossel, D. A. A., M. Visser, and A. M. R. Cornelissen. 1963. The examination of foods for *enterobacteriaceae* using a test of the type generally adopted for the detection of *salmonellae*. J. Appl. Bacteriol. **26**:444–452.
19. Savin, J. A. 1967. The microbiology of topical preparations in pharmaceutical practice. 1. Clinical aspects. Pharm. J. **199**:285–288.
20. Schothorst, M. van, D. A. A. Mossel, E. H. Kampelmacher, and E. F. Drion. 1966. The estimation of the hygienic quality of feed components using an *Enterobacteriaceae* enrichment test. Zentr. Veterinaermed., Reihe B, **13**:273–285.
21. Straka, R. P., and J. L. Stokes. 1957. Rapid destruction of bacteria in commonly used diluents and its elimination. Appl. Microbiol. **5**:21–25.