Tentative Method for the Qualitative Detection and Quantitative Assessment of Air Contamination by Drugs

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A method for detecting and measuring air contamination by drugs is described which uses an electrostatic bacterial air sampler, sprayers for micronizing drugs, and Mueller-Hinton medium seeded with a highly susceptible strain of Sarcina lutea. Three antibiotics (penicillin, tetracycline, aminosidine) and a sulfonamide (sulfapyrazine) were identified by pretreating portions of medium, showing no bacterial growth, with penicillinase or p-aminobenzoic acid solution and subsequently determining how both drug-susceptible and drug-resistant strains of Staphylococcus aureus were affected by this pretreatment. Quantitative determinations were also attempted by measuring the size of the inhibition zones.

Clinical experience and advances in control techniques have demonstrated that drugs containing traces of penicillin can be responsible for anaphylactic reactions, and at present the morbidity and mortality rates due to these anaphylactic reactions warrant the measures that have been adopted, or are being contemplated, for avoiding the dangerous occurrence of “penicillin cross-contamination in drugs.” Techniques are available whereby microbiological assays may be used for detecting even extremely minute amounts of penicillin contained in other drugs, such as, for instance, 0.005 unit in 1 g of a different drug (5). Our main problem today, therefore, is not how to detect such small quantities of penicillin but rather how to prevent them from contaminating other drugs during the complex processes of drug manufacture.

From our experience, as well as from that of other researchers (2–4), it has been found that air is a major vector of contaminants in drug production rooms. Recent technological progress in air purification methods, such as those based on the laminar flow principle, has been remarkable, and air can now be cleaned of all but a few of the microscopic particles it contains. A new method has been developed for counting particles endowed with antibacterial activity, and for giving a tentative indication as to whether any specific particle is made up of penicillin, tetracycline, aminosidine, or a sulfonamide.

MATERIALS AND METHODS
Initially the sensitivity was investigated by using the well-known bacterial strains Sarcina lutea ATCC 9341 and Staphylococcus aureus ATCC 6538P (1), and then we reused three strains of the latter organism which had become resistant to the different drugs being assayed. The organisms were first tested for sensitivity by using commercially available Sulfadiazine (Difco) and Unidisk (Difco) antibiotic no. 1 discs (low concentration), as well as paper discs of 6-mm diameter which had been soaked with known quantities of drug solutions and then allowed to dry on stainless-steel wire mesh in circulating air. Each disc of the latter contained 0.003 to 5 μg of penicillin G, tetracycline hydrochloride, aminosidine sulfate, or sulfapyrazine (Kelfazine). The strains were seeded on the surface of single, 5-mm thick layers of Mueller-Hinton medium (MH) contained in petri dishes, and full-depth plugs of the medium were removed by using sterile hollow punches after 5 hr of refrigeration at 5 C and 18 hr of incubation at 35 C. These 6-mm diameter culture medium plugs were then transferred to “reveler” type petri dishes, each containing a 3-mm layer of MH medium which had been seeded with freshly prepared broth cultures of S. aureus strains. Some of the plugs were treated before the assay for 4 hr at room temperature with (i) 0.05 ml of sterile penicillinase (Penase, Difco), (ii) sterile 0.1% p-aminobenzoic acid (PABA) solution, or (iii) sterile distilled water.

To make sure that the culture medium itself was without antibacterial activity, several portions were removed from the uninoculated culture medium and assayed by the method previously described.

When these preliminary tests had been completed, air contamination assays were initiated with
an electrostatic bacterial air sampler (EBAS; Gardner Associates Inc., Schenectady, N.Y.) normally employed for sampling known volumes of air and counting the microorganisms which are entrapped on the surface of agar dishes as they cross the sampler’s 7,000-v electromagnetic field. The experiments were carried out under the assumption that drug particles contained in the air would likewise be attracted by the electromagnetic force and that the petri dishes seeded with the test strains would permit the identification and subsequent quantitative determination of the particles.

The assays were carried out with the EBAS encased in a 1-m³ glass cabinet in which a pressure slightly above that of the room level was maintained, and gloves and sleeves were constantly used for handling the material from the outside. The same equipment settings and the operating sequences were used throughout the assays, and the air was removed from the cabinet after testing.

Each test, which was carried out on two petri dishes, lasted 8 min, equivalent to a total of 98 liters of air. Known quantities of micronized penicillin, tetracycline, aminosidine, or sulfapyrazine were sprayed together with 12/114 propellant for 20 sec during each test, and incubation was carried out at 35 C for 18 hr. Plugs of the medium, subsequently removed from the inhibition zones, were treated and assayed as previously described.

RESULTS

The sensitivity of six bacterial strains to different drugs is shown in Table 1. The inhibition zone diameters with Unidisks (Difco) averaged from four determinations (Table 1) show that there are different levels of sensitivity to individual drugs and that S. lutea is generally more sensitive to a greater number of drugs than are the other strains.

Table 2 shows the results of tests performed on S. lutea and S. aureus with decreasing amounts of the drugs. These tests have confirmed the high sensitivity of S. lutea even to sulfapyrazine and indicate the possibility of detecting amounts as small as 0.004 μg of penicillin G; therefore the strain was used for subsequent air assays.

Figure 1 shows the sensitivity of S. lutea to several drugs and the holes punched in the inhibition zones. The remarkable specificity of the test is illustrated in Fig. 2, which shows a “revealer” petri dish seeded with S. aureus and assayed with plugs of treated medium. Figure 3 shows two petri dishes, one seeded with S. lutea and exposed to penicillin spray, the other seeded with S. aureus ATCC 6538P and assayed as described (“revealer” petri dish).

Additional tests in which tetracycline, aminosidine, or sulfapyrazine sprays were used demonstrated the possibility of identifying the drugs sprayed by using homologous resistant strains of S. aureus. Conventional values were employed (Table 3) to make individual test results comparable. Average results and confidence limits (P = 0.05) of 13 tests in which penicillin aerosols were used, with or without pressurized air in the cabinet, are shown in Fig. 4 (for which the same scheme was used as outlined in Table 3). The antibacterial activity was at its peak during the spraying of the antibiotic. Traces of penicillin could be detected up to 20 min after spraying ended (when air in the cabinet was pressurized) or for 40 min when it was not.

The dynamics of the two tests appear to follow the same trend; the lines in the diagram are virtually parallel. The lower quantity of penicillin in the pressurized cabinet (line a) was due to a sizable portion of the antibiotic

| Table 1. Sensitivity of six test strains to various drugs (Difco Unidisk, antibiotic no. 1, low concentration) and sulfadiazine (50 μg) |
|-----------------------------------------------|
| Test strains                               | Diameter of inhibition zonea (mm) |
|                                            | Penicillin | Erythromycin | Tetracycline | Novobiocin | Neomycin | Kanamycin | Chloramphenicol | Dihydrostreptomycin | Sulfadiazine |
| Sarcina lutea ATCC 9341                     | 50.2       | 44           | 33.5         | 31.3       | 23        | 16.4       | 32.2           | 15               | 37           |
| Staphylococcus aureus ATCC 6538P            | 39         | 38.1         | 29.2         | 28         | 15.1      | 17.2       | 25.1           | 12               | 19.4          |
| S. aureus penicillin-r                      | 0          | 32.2         | 33.4         | 33         | 20        | 26         | 26.5           | 25.2             | 24           |
| S. aureus tetracycline-r                    | 30.1       | 22.3         | 0            | 40.5       | 38        | 35.4       | 41             | 40.3             | 30.1          |
| S. aureus aminosidine-r                     | 43.2       | 40.1         | 44.2         | 40         | ±         | ±          | 46.4           | (20)             | 30.8          |
| S. aureus sulfonamide-r                     | 48.4       | 43           | 40.5         | 38         | 37        | 40.2       | 36.6           | 40               | 0            |

*a Average of four replicate determinations; ( ), outline of areas not well defined; ±, trace of inhibition.

*b Micrograms of drug per disc.

c r denotes resistant strains.
Table 2. Sensitivity of Sarcina lutea (ATCC 9341) and Staphylococcus aureus (ATCC 6538P) to some drugs contained in freshly prepared paper discs

| Micrograms per disc | Penicillin G | Tetracycline | Aminosidine | Sulfapyrazine |
|---------------------|--------------|--------------|-------------|---------------|
|                     | S. lutea     | S. aureus    | S. lutea    | S. aureus    |
| 5.0                 | >50<sup>a</sup> | >50          | 32.2        | 29            |
| 2.5                 | >50          | >50          | 29.5        | 24.2          |
| 1.2                 | >50          | 42.4         | 27.5        | 21            |
| 0.6                 | 50           | 36           | 24.1        | 17.2          |
| 0.3                 | 43.2         | 30.2         | 22.5        | 13.5          |
| 0.15                | 40           | 24           | 19          | 0             |
| 0.075               | 33.2         | 18.4         | ±           | 0             |
| 0.032               | 28.5         | 12.1         | 0           | 0             |
| 0.016               | 24.4         | 0            | 0           | 0             |
| 0.008               | 20           | 0            | 0           | 0             |
| 0.004               | 16.2         | 0            | 0           | 0             |
| 0.002               | ±            | 0            | 0           | 0             |
| 5 + PASE<sup>c</sup> | 0            | 0            | 33.2        | 30            |
| 5 + PABA<sup>c</sup> | 50.4         | 36           | 32          | 28            |

<sup>a</sup> Average diameters of the inhibition zones (mm) of four replications are reported; ±, trace of inhibition.

<sup>c</sup> PASE, penicillinase.

<sup>c</sup> PABA, p-aminobenzoic acid.

Fig. 1. Sensitivity of Sarcina lutea to Unidisk (antibiotic no. 1, Difco, low concentration) and sulfadiazine (50 µg).

Fig. 2. Sensitivity of Staphylococcus aureus to drug contained in culture medium plugs removed from inhibition zones created by penicillin G (Unidisk). Plugs were treated with distilled water (top left), p-aminobenzoic acid solution (top right), and penicillinase (bottom right); the fourth plug was taken from a zone showing bacterial growth.

adhering to the cabinet walls, as was demonstrated by the Ten Cate method (6) with medium seeded with S. lutea.

Similar results were obtained with the other drugs. Tetracycline was detected for 20 and 30 min, respectively, aminosidine for 15 and 25 min, and sulfapyrazine for 15 and 20 min. Tests performed by spraying penicillin simultaneously with tetracycline or aminosidine showed that the presence of penicillin could be inferred from the plugs treated with penicillinase exhibiting smaller inhibition zones than those treated with distilled water.

The minimal appreciable quantities of penicillin, tetracycline, aminosidine, and sulfapyrazine sprayed with no pressurized air in the cabinet were 0.3, 1.4, 2.0, and 4.4 µg, respectively, per m³ of air. Amounts of antibacterial substances in the air could be fairly accurately determined by measuring the extent of antimicrobial areas.
FIG. 3. Left: Sarcina lutea dish after exposure to penicillin spray. Right: Staphylococcus aureus dish assayed against culture medium plugs removed from an antimicrobial zone from the first plate. Plugs were treated with distilled water (bottom left), p-aminobenzoic acid solution (top left), and penicillinase solution (top right); the fourth plug was taken from a zone showing bacterial growth.

TABLE 3. Conventional scheme of measurement adopted in the experiments

| Code of inhibition zone | Range of inhibition zone diameter (mm) | Inhibition area (mm²) |
|-------------------------|----------------------------------------|-----------------------|
| Small                   | 2–4                                    | 7                     |
| Medium                  | 5–10                                   | 45                    |
| Large                   | 11–25                                  | 250                   |
| Half of petri dish      |                                        | 2,600                 |
| Whole petri dish        | 80                                     | 5,200                 |

DISCUSSION

Drug particles were identified by the same method used for determining antibiotics diffused in agar from paper discs (sensitivity test), i.e., by testing the plugs of medium with strains sensitive or resistant to known drugs. The treatment of the plugs with Penase or PABA permits one to identify the presence of penicillins or sulfonamides. The use of an electrostatic air sampler is not indispensable; similar equipment, with or without an electromagnetic field, may definitely be employed.

By using such an air sampler and S. lutea as the identifying strain, antibiotic amounts as small as 0.3 µg of penicillin G or several micrograms of tetracycline, aminosidine, or sulfapyrazine per cubic meter of air could be detected. An important finding was that such small quantities of the drugs were still detectable in the air 20 to 40 min after the drugs had been sprayed on. This could be of considerable importance chiefly in drug manufacturing plants, where different pharmaceuticals may be processed consecutively. The method described could no doubt be used for detecting other antimicrobial drugs besides the ones considered in our research, as long as the homologous resistant strain is available. When qualitative tests are performed, care should be exercised in using the proper drug-resistant strains, and the possibility should not be overlooked of interference from drug excipients and of cross-reactions occurring between individual antibiotics.

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