Biotyping of Enterobacteriaceae as a test for the evaluation of isolation systems

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SUMMARY

Arguments in favour of biotyping of Enterobacteriaceae excreted in the faeces of isolated patients, as a method of investigating the efficiency of the isolation procedures, are presented as well as a technical outline of the procedure. The study included three kidney transplantation patients, five acute myeloid leukaemia patients and four healthy persons as controls.

The results show, apart from new colonizations during isolation, a difference in the mean number of contaminations and colonizations with different Enterobacteriaceae biotypes. It is concluded from these results, that the isolation procedures were not completely effective and that the AML patients studied had a decreased colonization resistance of their digestive tract. This was less evident in the kidney transplant group.

INTRODUCTION

With the growing interest in the application of reverse isolation systems for patients, the necessity increases for an adequate test-system to evaluate the efficacy of the isolation procedures. In the literature, two approaches have been described: the use of germ-free mice (Barnes, Tuffery & Cook, 1968; Meindersma & Van de Waaij, 1968) and the typing of Staphylococcus aureus and Pseudomonas aeruginosa obtained from swabs or other samples from isolated patients (Jameson, Gamble, Lynch & Kay, 1971; Lidwell & Towers, 1972). The maintenance of germ-free mice in an isolator is, in fact, only a test of the quality of the isolator and not necessarily of the isolation system. The latter involves, apart from an isolator, all precautions taken to prevent contamination of the inside of the unit. Typing of staphylococci isolated from the patients seems much more adequate. Isolation of staphylococci of a different phage type than was found at admittance, may indicate the occurrence of contaminations in an isolated patient. In a properly functioning isolation system, such contaminations should not occur. Schneider et al. (1969) express the efficacy of isolation by comparing the incidence of infections in isolated patients with that in similar patients undergoing similar immuno-
suppressive treatment without isolation. We feel that this is a questionable criterion since it is influenced by many factors apart from protective isolation.

A great majority of the infections seen in patients with a decreased resistance against infections, however, are caused by Gram-negative rods (Hersh, Bodey, Nies & Freireich, 1965). These bacteria, *Pseudomonas aeruginosa* and *Enterobacteriaceae* species, have a different epidemiology than staphylococci. It is, for example, possible to select the human contacts of a patient on the basis of 'carriage of *Staphylococcus aureus*'. Nurses are excluded for some time from helping isolated patients should they carry *Staph. aureus*. This holds also for *Ps. aeruginosa* (Schneider et al. 1969). This species is more frequently involved in dangerous infectious complications in patients with decreased immunocompetence than is *Staph. aureus* (Hersh et al. 1965). Selection of the medical personnel or other human contacts for an isolation unit on the basis of 'carriage of Enterobacteriaceae species' is impossible, since everyone is colonized by one or more of these species.

These considerations have also been found to be applicable to bone marrow transplantation experiments in monkeys. About 3 years ago, a typing procedure was sought for the typing of Enterobacteriaceae species in isolated *Macaca mulatta*. Serological typing was discarded, since (except for salmonellas and shigellas) it is mainly confined to two Enterobacteriaceae species: *Escherichia coli* (Kauffmann, 1947) and representatives of the family of *Klebsiella* (Orskov, 1952, 1954). Secondly, it is a very laborious technique. The possibility of 'biotyping' was therefore investigated. With this technique, the isolation precautions taken in the treatment of lethally irradiated monkeys could be investigated and improved (Van der Waaij, unpublished data).

Recently, this approach was also applied to patients treated in the Unit for protective isolation at the University Hospital of Leiden. In two groups of patients and in four healthy controls an inventory was made at the onset of the study. Subsequently, the occurrence of contaminations (if a biotype was only isolated once) as well as of colonization (if a biotype was found in several subsequent samples from the same patient) with Enterobacteriaceae species, was investigated. To this end, at least twice a week, faecal samples were processed for pure culturing of at least 20 Enterobacteriaceae colonies per sample. These were then biotyped. One group of five patients consisted of acute myeloid leukemia (AML) patients. The other consisted of three patients treated with immunosuppressive drugs after kidney transplantation. The term 'contamination' will be used in this report to describe the situation where the patient acquires such a number of bacteria of a certain biotype that a 'take' occurs. This implies that for a short period of 1 or 2 days, a concentration of at least $10^4$ cells/g. is reached in the faeces of that patient. The term 'colonization' will be used in those cases where a biotype persists in the patient for a time interval longer than 4 days.

**MATERIALS AND METHODS**

*Sampling*

The investigation period in the eight patients involved in this study depended on the duration of their stay in the isolation ward, but was at least 2 weeks. The
### Table 1. Tests used in biotyping Enterobacteriaceae

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Test</th>
<th>Serial number</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Xylose</td>
<td>11</td>
<td>Dulcitol</td>
</tr>
<tr>
<td>2</td>
<td>Maltose</td>
<td>12</td>
<td>Ornithine decarboxyl.</td>
</tr>
<tr>
<td>3</td>
<td>Rhamnose</td>
<td>13</td>
<td>Lysine decarboxyl.</td>
</tr>
<tr>
<td>4</td>
<td>Mannitol</td>
<td>14</td>
<td>H₂S</td>
</tr>
<tr>
<td>5</td>
<td>Arabinose</td>
<td>15</td>
<td>Urease</td>
</tr>
<tr>
<td>6</td>
<td>Sorbitol</td>
<td>16</td>
<td>Citrate</td>
</tr>
<tr>
<td>7</td>
<td>Lactose</td>
<td>17</td>
<td>Inositol</td>
</tr>
<tr>
<td>8</td>
<td>Indol</td>
<td>18</td>
<td>Adonitol</td>
</tr>
<tr>
<td>9</td>
<td>Sucrose</td>
<td>19</td>
<td>Raffinose</td>
</tr>
<tr>
<td>10</td>
<td>Salicine</td>
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</table>

Four healthy controls were followed for 6 weeks. Faeces samples were collected twice a week from the patients as well as from the controls. In most cases, faecal samples of 1–2 g. were either directly processed, or stored for some time in liquid nitrogen. Fresh faecal material, as well as material that had been stored in liquid nitrogen, was suspended (1/10) in brain heart infusion (BHI) broth (DIFCO). Directly, and after incubation for 4 hr. at 37°C, subinoculation onto Endo-agar (DIFCO) was performed. The faeces were also streaked directly onto Endo-agar after suspending in saline in a dilution of 1/10.

**Isolation of pure cultures**

After 24 hr. incubation of the Endo plates, at least 20 (where possible) morphologically different colonies (per faecal sample) were picked from the Endo-agar and subinoculated back onto fresh Endo-agar. Each colony received its own serial number (1 to 20) which was not changed until the typing had been completed. Pure culturing was continued after incubation of the second series of Endo-agars on Kligler slants (DIFCO). On this medium the ‘purity’ of the cultures was carefully checked. In addition, the Kligler slants gave the results of two biochemical reactions: fermentation of lactose and formation of H₂S.

**Fermentation reactions**

Most of the fermentation reactions (Table 1) were performed in plastic trays with 64 cups of 2 ml. volume. For this purpose the technique and the media described by Guinee, van Leeuwen & Jansen (1972) were used. Tests for decarboxylase and urease production were performed in the same plastic trays. These cups, however, were sealed after inoculation. Inoculation of cups was performed with two drops of a saline suspension consisting of approximately 10⁸ bacteria/ml. Such suspensions were prepared from each pure culture on the Kligler slants. To prevent contamination and dehydration of the media during incubation, the trays were incubated inside well closed and disinfected plastic boxes.

**Reading of the tests**

The tests were read after 24 hr. of incubation at 37°C. Reading after longer time intervals gave different and less constant results. It should be mentioned here
that, in several cases, the classical routine bacteriological results were not obtained. ‘Slow lactose fermenters’, for example, are listed as ‘positive’ in routine bacteriology. Twenty-four hours incubation, however, has mostly been too short in our typing system for these strains to demonstrate this capacity. For typing purposes, we were not interested in strain identification, but in constancy of the results. Each strain, when tested repeatedly, should give identical results in the 19 reactions selected. Use of this system in monkeys had indicated that, with a few exceptions, these biotype characteristics are stable properties of Enterobacteriaceae.

Coding of the results

The results were coded by use of the binary system. The strains were thus identified by a number generated by the formula

\[ \sum_{i=1}^{20} a_i \times 2^{20-i}, \]

in which \( i \) = code number of the test (Table 1), \( a_i = 1 \) if the \( i \)th test is positive and \( a_i = 0 \) if the \( i \)th test is negative. For example: if the tests with the serial numbers 1, 2, 12, 13 and 16 were positive, the biotype of this particular strain would be

\[ 2^{20-1} + 2^{20-2} + 2^{20-12} + 2^{20-13} + 2^{20-16} = 786,832. \]

Decoding and ‘translation’ of the biotypes back to the standard bacteriological nomenclature is obviously possible at any time, but this is unnecessary in epidemiological studies.

Method of determining whether as many as possible different Enterobacteriaceae biotypes had been isolated per sample

Twenty Enterobacteriaceae colonies per sample were typed as a minimum. Whether or not a sufficient number of morphologically different colonies had been typed to allow a good inventory of an individual was investigated by giving the isolates a serial number. The number of different biotypes found in the first group of four colonies was then plotted at the 4th place of the abscissus. The number of biotypes differing from the first four and from each other in the second group of four colonies typed is plotted at the 8th place on the abscissus, etc. (Fig. 1). A good insight into the adequacy of the number of colonies typed was then obtained by drawing a curve through these points. If this curve reached the abscissa in the 5th group of four (numbers 17, 18, 19 and 20) it was assumed that a sufficient number of colonies had been typed. If the curve did not reach the abscissa in the 5th group, more colonies were isolated from the original Endo plates that had been stored in the refrigerator. The number of subsequent isolates depended on the slope of the curve found with the first 20 colonies (Fig. 1).

Test for minimum detectable concentration of Enterobacteriaceae biotypes in faeces

For this purpose, suspensions of streptomycin-resistant strains of \textit{E. coli}, \textit{Proteus mirabilis} and \textit{Klebsiella pneumoniae} were mixed in different concentrations with human faeces free of streptomycin-resistant Enterobacteriaceae species. The biotypes of these antibiotic resistant strains were known, the resistance to streptomycin gave the strains an additional marker. The concentrations obtained in the
faeces varied between $10^3$ and $10^7$ bacterial cells per g. Isolation of the ‘test strains’ from these mixtures was performed in two ways: (1) the method described above; and (2) the faecal material was streaked onto Endo-streptomycin agar (2 mg/ml).

**Isolation system for patients**

The isolation facilities and the precautions used have been described elsewhere in detail (Vossen & Van der Waaij, 1972). The patients are maintained in single-bed rooms provided with a personnel ante-room as well as an entry lock for materials. The rooms are mechanically ventilated with filtered air (unipack AAF) providing nine changes/hr. The rooms are well disinfected before use. All items, except food and beverages, are double wrapped and sterilized by steam or ethylene oxide. Food and beverages are prepared in the kitchen of the Unit. Each room has a sink and is provided with a toilet that can be reached via the personnel ante-room.

**Antibiotics**

The antibiotics used for the therapy of infections in the isolated patients were:

- cephaloridin (parenteral)
- gentamycin (parenteral)
- rifampicin (parenteral)
- celbenin (parenteral)
- ampicilin (parenteral)
- orbenin (oral)
- acipen (oral)

The sensitivity pattern of ‘colonizing’ biotypes was investigated on BHI-agar with antibiotic disks consisting of the following antibiotics: streptomycin, kanamycin, ampicillin, carbenicillin, terramycin, chloramphenicol.
RESULTS

Technical

The minimum detectable concentration of Enterobacteriaceae species in the faeces appeared to be about $10^4$ bacteria/g. Both by biotyping and on the Endo-streptomycin agar, the Enterobacteriaceae species mixed with the faeces were recovered from six out of nine samples containing $10^4$ cells/g. and from all higher concentrations. Since the sample size for biotyping was approximately 1 mg., it is to be expected that samples containing $10^3$ cells/g. or less, would not yield sufficient colonies for our purposes.

The number of colonies to be picked from an Endo according to the curve (Fig. 1) was plotted against the number of different biotypes found per faecal sample (Fig. 2). The correlation between the number of isolates and the number of different biotypes appeared to be rather constant (S.E. = 0.060).

Patients

During their stay in the Unit, five patients had infections and were treated with antibiotics. Both the oral and the parenteral route of administration was used. The first turned out to have an interesting influence on the contamination and colonization (C and C) pattern. In Fig. 3, the ‘C and C-pattern’ of a representative healthy control is depicted, whereas Figs. 4 and 5 show the C and C-patterns of a representative kidney transplantation patient and an AML patient respectively.

The mean number of ‘contaminations’ seen in the unisolated healthy controls was found to be lower (mean number less than 2) than in the isolated AML-patients (mean number between 3 and 6) (Fig. 6). The isolated kidney transplanted patients were more of the control type in this respect. It is also seen that the mean number of contaminations that resulted in colonization for some time, was somewhat higher in the AML patients than in both other groups (Fig. 6). The influence of oral and systemic antibiotic treatment on the ‘C and C pattern’ is given in Table 2. Systemic antibiotic treatment appeared to have no significant influence. Oral
Biotyping of Enterobacteriaceae

Fig. 3. Results of biotyping of Enterobacteriaceae species of faecal samples from a healthy control (not isolated).

Fig. 4. Results of biotyping of Enterobacteriaceae species of faecal samples from a kidney transplantation patient (isolated).

treatment for which only penicillins like acipen and orbenin were used, strongly increased the number of contaminations.

Similar results have been obtained in monkeys (van der Waaij, unpublished data). An increase in the mean number of contaminations and in some degree of colonization was seen in all nine animals studied, during the period of decreased immune-competence. This was the more evident, the less the precautions taken to prevent
contaminations. The influence of antibiotic treatment has also been clearly demonstrated in monkeys.

The occurrence of cross-contaminations with Enterobacteriaceae species between patients was our next concern. During the period of March, April and part of May
Table 2. Mean number of contaminations and colonizations in six isolated acute myeloid leukemia patients during antibiotic treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean number of contaminations</th>
<th>Mean number of colonizations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral antibiotic</td>
<td>5.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Systemic antibiotic</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>No antibiotic treatment</td>
<td>3.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 3. 173 contaminations in five patients during 135 isolation days in the period March–April 1971

- 146 occurring in one patient at a time
- 23 occurring in two patients at a time
- 2 occurring in three patients at a time
- 2 occurring in four patients at a time

In 1971, five patients involved in this study were isolated, either with an overlap or simultaneously. In these patients, identical biotypes had been observed on several occasions (Table 3). In about 50% of the cases, a certain biotype was isolated from two patients in the same week. This may indicate that both had been contaminated by the same primary (food?) source. In the other 50%, however, cross-contamination between patients could not be excluded because of an interval of at least 1 week between the isolation of the same biotype from two different patients. As in observations described by Selden et al. (1971), and Winterbauer, Turck & Petersdorf (1967), only ‘colonizing biotypes’ appeared to be involved in infectious processes in the patients. In the three kidney transplantation patients, only biotypes were involved that were also found at admission. This was different in the AML-patients. In this group, in two out of four cases, biotypes that colonized the patient for the first time during isolation were involved.

Colonizing biotypes were also submitted to an antibiotic sensitivity test. All 16 strains thus tested were found to have a normal sensitivity pattern. This may indicate that the ‘leak’ found in the barrier of the isolation system was not one from the general hospital outside, since many resistant Enterobacteriaceae species are known to circulate there.

**DISCUSSION**

The results of this study have shown that the biotyping technique is applicable for testing the efficiency of isolation systems. Incidental airborne contaminations of the tray in the laboratory may have influenced the result of biotyping in that it ‘changed’ a biotype in making one of the fermentation reactions falsely positive. We feel however, that, provided the test is performed very carefully and precisely, particularly with regard to pure culturing, biotyping of Enterobacteriaceae gives a good insight into the efficiency of the various precautions taken to prevent exogenous infections. Even if one should now and then falsely report a new biotype, which is due to a false positive test, this will not influence the conclusion which is based on the mean number of contaminations and colonizations. With regard to
isolation, it should be mentioned that only colonizing strains are potentially
dangerous with respect to causing infections (Kessner & Lepper, 1967). Secondly,
only an increase in the mean number of contaminations recorded in a period of at
least two weeks, should be considered as an indication that the Colonization Resist-
ance (CR) (Van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees, 1971)
of the digestive tract of a patient has decreased.

Patients suffering from AML, being remarkably susceptible to infections, all
showed an increased mean number of contaminations with Enterobacteriaceae
biotypes. This may indicate that they also had a decreased CR, which may have
enhanced the risk of colonization by an infectious agent. Colonization by Enterob-
eriaceae species, however, was apparently limited to biotypes that were
introduced into the isolation ward from sources other than the general ward.

In the AML patients more contaminations and colonizations were observed than
in healthy controls. The Contamination–Colonization incidence in the kidney
transplantation patients under immunosuppressive treatment however, was more
of the type of the healthy controls, than of the AML group of patients. Immuno-
suppression performed in both groups of patients, under conditions provided at
this Isolation Unit, apparently does not necessarily adversely influence the Colon-
ization Resistance. Oral treatment with penicillins, however, significantly in-
creased the number of colonizations during isolation. Possibly, the factors
controlling the occurrence of contaminations are more strongly influenced by oral
penicillin treatment than are those controlling colonizations. A more likely
explanation, however, seems to be one that takes into account an asymmetric
distribution of the numbers of bacteria of the various biotypes involved in con-
tamination and colonization. Because of all precautions taken to prevent con-
tamination, in the Isolation Pavilion, the peak occurrence of contamination will
have been in the very low dose range. Assuming that, as in mice, higher numbers
of bacteria are required for colonization than for contamination (Van der Waaij
et al. 1971), the large majority of contaminations may have gone unnoticed in
isolated AML patients. When, however, the CR was significantly decreased by
the oral penicillin treatment, several of these ‘low dose contaminations’ may have
taken for a few days, reaching the minimally detectable concentration in the
faeces of $10^4$ cells/g. In the biotyping procedure they were then recorded as a
contamination. If the dose distribution had indeed been asymmetrical only a few
biotypes in the higher dose contaminations may have moved into the colonization
category (which requires relatively higher doses).

Isolation of patients with a strongly decreased defence capacity in single-bed
rooms under isolation conditions appeared to be more complicated than was
expected. The relatively high incidence of contaminations and colonizations seen
in the isolated patients indicates a potential chance of contamination with more
pathogenic and more resistant Gram-negative rods. Most of the contaminating
Enterobacteriaceae species isolated were, presumably, of low pathogenicity. All
colonizing exogenous strains had a good sensitivity pattern. This may explain the
fact that, in the patients studied, most of the exogenous colonizing biotypes did
not cause infections.
Biotyping of Enterobacteriaceae

Others have described similar experiences with comparable isolation systems. Jameson et al. (1971) mentioned the occurrence of a flaw in kitchen procedure as the source of contaminations with a biochemically atypical coliform. They also describe an outbreak of Pseudomonas colonizations which could be traced to a nurse carrying this phage type of Pseudomonas. In our system, we also have indications that both the food and the staff may have been the main contamination sources. The personnel did not wear gloves during handling of the patients or in preparing the food. Solberg et al. (1971) described a very successful isolation of a patient in a laminar flow room. The latter will have only prevented airborne contamination. All materials entering the room, including food, were double wrapped and sterilized. The staff were well shielded wearing sterilized hoods, coats, calf-length boots, face masks, and gloves. Applying the same type of isolation, Vossen & Van der Waaij (1972) also reported similar successful isolation of several patients.

After this investigation was completed, the ventilation system of the isolation rooms was improved. Preparation of sterile food and beverages is now performed under strict aseptic conditions, while handling of the patients is performed with sterile gloves. Use of the toilet by the patient is no longer permitted. Instead, sterilized disposable bed-pans are employed. The sink has been modified so as to make effective daily disinfection possible. Under these isolation conditions, the study will be repeated.

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REFERENCES


