639

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

By D. van der WAAIJ and T. M. SPELTIE

Radiobiological Institute TNO, Lange Kleiweg 151,
Rijswijk (Z.H.), The Netherlands

and J. M. VOSSEN

Isolation Pavilion of J. A. Cohen Institute for Radiopathology and
Radioprotection, University Hospital, Leiden, The Netherlands

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 Entero-bacteriaceae 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* (Ørskov, 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*

Serial code number	Test	Serial code number	Test
1	Xylose	11	Dulcitol
2	Maltose	12	Ornithine decarboxyl.
3	Rhamnose	13	Lysine decarboxyl.
4	Mannitol	14	H_2S
5	Arabinose	15	Urease
6	Sorbitol	16	Citrate
7	Lactose	17	Inositol
8	Indol	18	Adonitol
9	Sucrose	19	Raffinose
10	Salicine		

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 H_2S.

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 Guinée, 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 10^8 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 ith test is positive and $a_i = 0$ if the ith 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 abscissus 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 abscissus 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 *E. coli*, *Proteus mirabilis* and *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
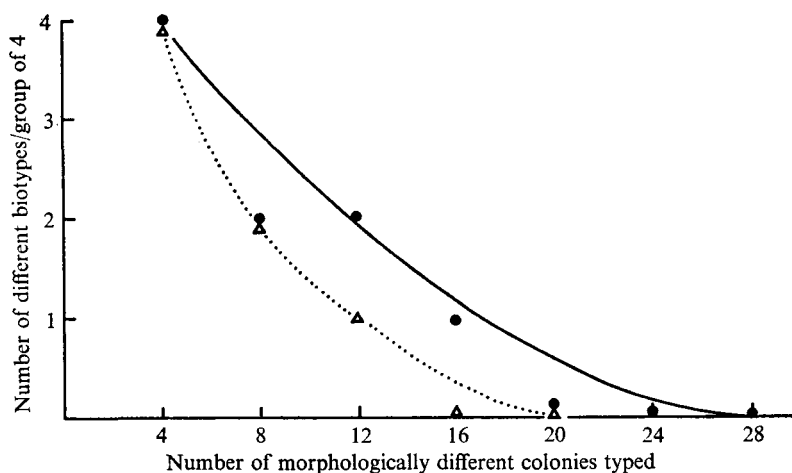
Fig. 1. Number of colonies required for a good inventory of the number of different Enterobacteriaceae biotypes in a faecal sample. The curves are shown of two representative cases: in one 20 colonies were sufficient, in the other eight more were required.

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

The sensitivity pattern of 'colonizing' biotypes was investigated on BHI-agar with antibiotic disks consisting of the following antibiotics: streptomycin, kana-mycin, ampicillin, carbenicillin, terramycin, chloramphenicol.
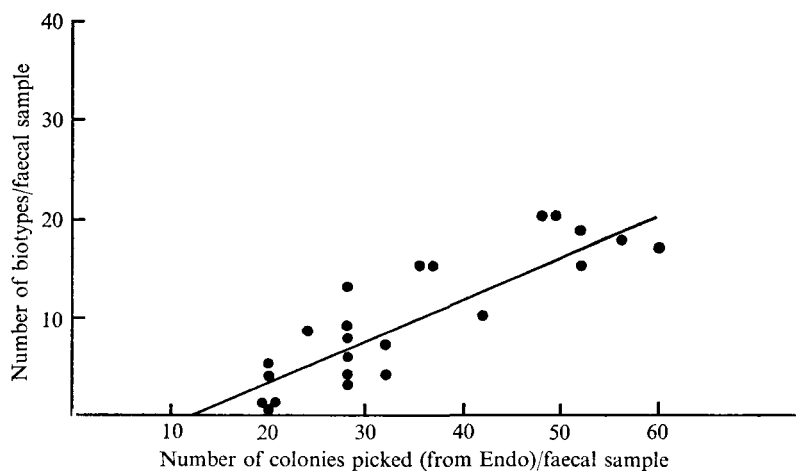
Fig. 2. Correlation between the number of different biotypes and the number of colonies picked from an Endo plate inoculated with faecal material.

RESULTS

Technical

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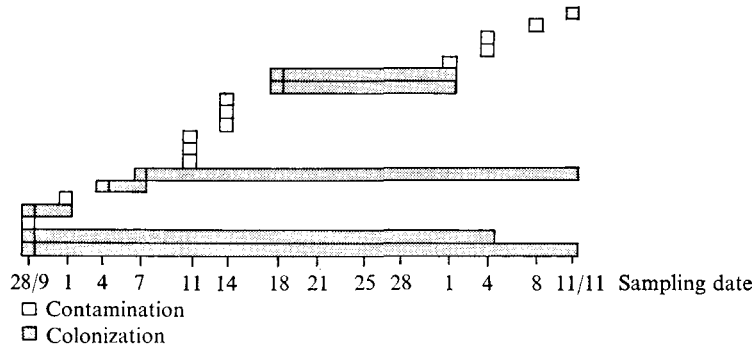
Fig. 3. Results of biotyping of Enterobacteriaceae species of faecal samples from a healthy control (not isolated).
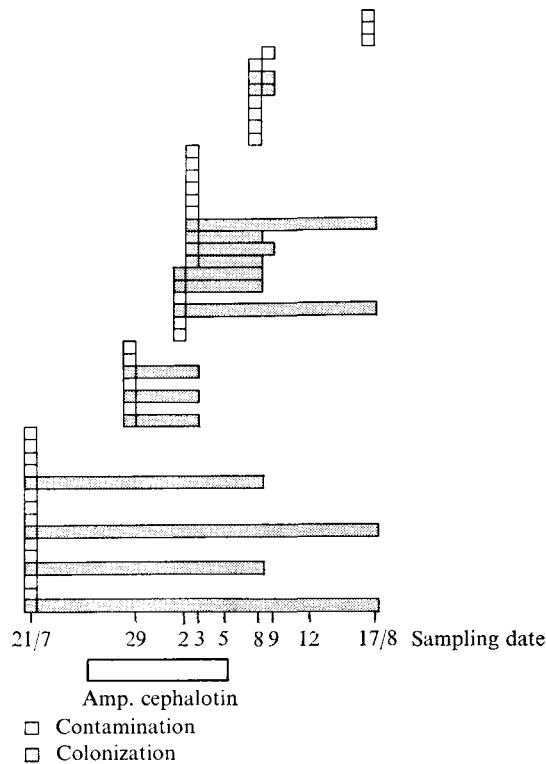

Fig. 4. Results of biotyping of Enterobacteriaceae species of faecal samples from a kidney transplantation patient (isolated).

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 immunecompetence. This was the more evident, the less the precautions taken to prevent
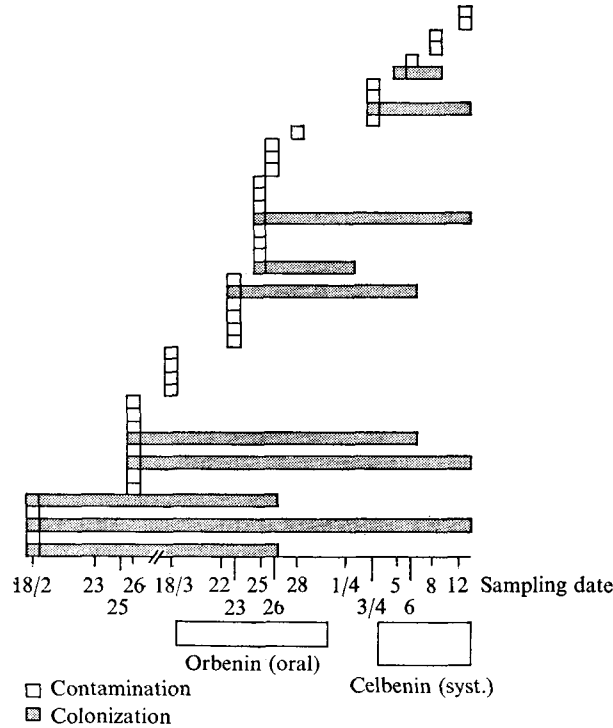
42　　　　　　　　　　　　　　　　　　　　　　　　　HYG 70

Fig. 5. Results of biotyping of Enterobacteriaceae species of faecal samples from an AML patient (isolated).
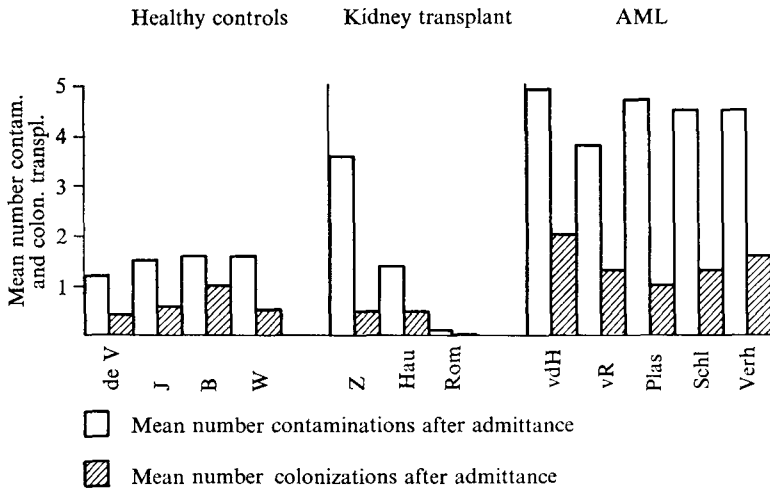

Fig. 6. Mean number of contaminations and colonizations after admission in the three groups studied.

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 leukaemia patients during antibiotic treatment*

Treatment	Mean number of contaminations	Mean number of colonizations
Oral antibiotic	5·1	0·9
Systemic antibiotic	3·4	1·3
No antibiotic treatment	3·3	0·9

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

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

42-2

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 Resistance (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 Enterobacteriaceae 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. Immunosuppression performed in both groups of patients, under conditions provided at this Isolation Unit, apparently does not necessarily adversely influence the Colonization Resistance. Oral treatment with penicillins, however, significantly increased 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 contamination and colonization. Because of all precautions taken to prevent contamination, 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.

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.

The authors are very grateful for the great hospitality encountered in the Isolation Pavilion of the J. A. Cohen Institute for Radiopathology and Radioprotection, Leiden; Director: Dr B. Speck.

REFERENCES

BARNES, R. D., TUFFERY, M. & COOK, R. (1968). A 'germfree' human isolator. *Lancet* i, 622.

DIETRICH, M., FLIEDNER, T. M. & HEIMPEL, H. (1969). Isolierbettsystem zu Infektionsprophylaxe bei verminderte Resistenz. *Deutsche Medizinische Wochenschrift* **94**, 1003.

GUINÉE, P. A. M., VAN LEEUWEN, W. J. & JANSEN, W. M. (1972). A new technique for the biotyping of *S. typhimurium*. *Applied Microbiology*, submitted for publication.

HERSCH, E. M., BODEY, Q. P., NIES, B. A. & FREIREICH, E. J. (1965). Causes of death in acute leukemia. *Journal of the American Medical Association* **193**, 99.

JAMESON, B., GAMBLE, D. R., LYNCH, J. & KAY, H. E. M. (1971). Five-year analysis of protective isolation. *Lancet* i, 1034.

KAUFFMANN, F. (1947). The serology of the coli group. *Journal of Immunology* **57**, 71.

KESSNER, D. M. & LEPPER, M. H. (1967). Epidemiologic studies of Gram-negative bacilli in the hospital and community. *American Journal of Epidemiology* **85**, 45.

LIDWELL, O. M. & TOWERS, A. G. (1972). Unidirectional ('Laminar') flow ventilation system for patient isolation. *Lancet* i, 347.

MEINDERSMA, T. E. & VAN DER WAAIJ, D. (1968). Observations on isolators for patients. *Folia medica Nederlandica* **11/3**, 76.

ØRSKOV, J. (1952). Nosocomial infection with *Klebsiella* in lesions of the urinary tract. *Acta Pathologica et Microbiologica Scandinavica, Supplement* **93**, 259.

ØRSKOV, J. (1954). Serological inventory of the *Klebsiella* group. *Acta Pathologica et Microbiologica Scandinavica* **34**, 145.

SCHNEIDER, M., SCHWARZENBERG, L., AMIEL, J. L., CATTAN, A., SCHLUMBERGER, J. R., HAYAT, M., DE VASSAL, F., JASMIN, C. L., ROSENFELD, C. C. & MATHÉ, G. (1969). Pathogen-free Isolation Unit – Three Years Experience. *British Medical Journal* i, 836.

SELDEN, R., LEE, S., WANG, W. L., BENNETT, J. V. & EICKHOFF, T. C. (1971). Nosocomial *Klebsiella* infections: Intestinal colonization as a reservoir. *Annals of Internal Medicine* **74**, 657.

Solberg, C. O., Matsen, J. M., Vesley, D., Wheeler, D. J., Good, R. A. & Meuwissen, H. J. (1971). Laminar airflow protection in bone marrow transplantation. *Applied Microbiology* **21**, 209.

Vossen, J. M. & Van der Waaij, D. (1972). Reverse isolation in bone marrow transplantation. II. Microbiological and clinical results. *European Journal of Cancer* (in the Press).

Waaij, D. van der, Berghuis-de Vries, J. M. & Lekkerkerk-van der Wees, J. E. C. (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *Journal of Hygiene* **69**, 405.

Winterbaner, R. H., Turck, M. & Petersdorf, R. G. (1967). Studies on the epidemiology of *Escherichia coli* infections. V. Factors influencing acquisition of specific serologic groups. *Journal of Clinical Investigation* **46**, 21.