Determination of colonization resistance of the digestive tract by biotyping of Enterobacteriaceae

H. Z. APPERLOO-RENKEMA, B. D. VAN DER WAAIJ
AND D. VAN DER WAAIJ

*Laboratory for Medical Microbiology, State University of Groningen,
Oostersingel 59, NL-9713 EZ Groningen, The Netherlands*

SUMMARY

In studies concerning the effect of antibiotics on faecal microflora, Colonization Resistance is an important parameter. Colonization Resistance correlates inversely with the number of different biotypes of Enterobacteriaceae isolated from faecal samples. Nine healthy volunteers were studied during 6 weeks, in order to determine the natural variation in the number of different biotypes of Enterobacteriaceae per faecal sample. The numbers of biotypes ranged from 1–15 per faecal sample, the mean number of biotypes varied between 2·6 and 7·3 different biotypes per faecal sample per healthy volunteer. Inter-individual variations of five biotypes in the mean number of biotypes per faecal sample are normal. We assessed the minimal number of faecal samples that should be taken for comprehensive biotyping so as to determine reliably the mean number of different biotypes representative for the Colonization Resistance of an individual. It was found that a minimum of four faecal samples was required.

INTRODUCTION

Studies of the effect of antibiotics on faecal microflora can use the Colonization Resistance (CR) as a parameter [1–3]. The CR of the digestive tract is mainly sustained by the indigenous anaerobic bacteria, by their numerous and very complex interactions. Newly ingested bacteria trying to colonize the digestive tract encounter great difficulty in doing so, probably because of competition for niches and nutriments with the indigenous predominantly anaerobic flora. Normally an equilibrium exists between the resident anaerobic flora and the aerobic flora of the digestive tract. The stronger the suppression of ingested bacteria by the anaerobic flora, i.e. the stronger the CR, the fewer different biotypes of Enterobacteriaceae are apparently able to colonize the gut. Enterobacteriaceae species, particularly, *Escherichia coli* biotypes, in general form an important part of the aerobic flora of the digestive tract. Biotyping of Enterobacteriaceae provide a valuable and reproducible method for differentiation below the species level. Some species can be subdivided in more than 50 different biotypes [4–6]. In mice, CR is defined as the resistance of the digestive tract against colonization by orally ingested potentially pathogenic bacteria [7]. The

CR of an individual mouse can be measured after oral contamination with a specific potentially pathogenic bacterial species and expressed as the log concentration of that species found in the faeces 2 weeks after contamination [8].

In humans, determination of the CR during antibiotic treatment is of clinical importance, particularly in immunocompromised patients. If large parts of the anaerobic flora are destroyed by antibiotic treatment, resistant non-indigenous bacteria may colonize the gastrointestinal tract in great numbers. The digestive tracts of individuals with an impairment of their CR are likely to become colonized in high concentrations by potentially pathogenic bacteria [9–11]. Dominant clones of Enterobacteriaceae, those that grow out to high numbers, represent the strains which are most likely to translocate to lymphatic organs [12]. Translocation of potentially pathogenic bacteria is expected to occur less frequently in patients with a high CR, and consequently low concentrations of these potentionally pathogenic microorganisms, than in patients with a low CR.

In immunocompromised patients translocation of Enterobacteriaceae may result in a life-threatening septicaemia [10, 11, 13]. In order to estimate the risk of infection in these patients, it is important to be able to determine the CR. In view of the risk of infection, however, it is unethical to contaminate (immunocompromised) patients orally with potentially pathogenic bacteria, as has been done in mice [8, 11], in order to determine their CR. It was therefore felt necessary to search for another way of assessing the CR.

In mice there is evidence for an inverse relationship between CR and the mean number of different biotypes of Enterobacteriaceae that can be isolated from the faeces [8]. Determination of the mean number of biotypes in faeces by comprehensive typing of Enterobacteriaceae is also possible in man.

We have analysed the data of a comprehensive biotyping study in healthy volunteers starting in 1971 and completed in 1982. The object of our analysis was to assess the natural variation in the number of different biotypes of Enterobacteriaceae found in the faecal samples of healthy volunteers. For practical reasons we also wanted to determine the minimum number of faecal samples which should be biotyped to calculate the mean number of different biotypes as a reliable measure for the CR. To avoid influences in the outcome due to changes in the laboratory staff as well as possible changes in the production of the biotyping system, the biotyping results of faeces of volunteers were taken over a long period. Except for 1972 a volunteer entered the study annually.

MATERIALS AND METHODS

Healthy volunteers

Nine healthy volunteers, seven male and two female, aged 22–51 years, participated in the study. None of them had taken antibiotics for 8 weeks prior to or during the experiment nor had they suffered any infective illness during the period they were on study.

Sampling

Two faecal samples were obtained every week from each volunteer for 6 weeks.

Biotyping

Faeces were inoculated directly onto MacConkey agar (Merck) and were additionally suspended 1:9 (w/v) in Brain Heart Infusion (BHI) broth (Oxoid). These faecal suspensions were then diluted 1:9 (v/v) in BHI broth. After incubation the various suspensions were subcultured on MacConkey agar. Enterobacteriaceae species were identified and typed with 19 different fermentation reactions selected for the Enterobacteriaceae. For comprehensive biotyping a minimum of 20 single colonies was cultured per sample. Details of the biotyping technique have been described previously [4].

Statistical analysis

Statistical software has been developed for data storage and processing of the results of the biotyping experiment.

The results are shown in a biotyping diagram as previously described [3]. Briefly: on the horizontal axis the different sequential samples were plotted while the number and the codes of the different biotypes were indicated along the vertical axis. Biotypes isolated in subsequent samples and identical to previously isolated ones, were plotted on the same level. Biotypes were plotted on the vertical axis according to their sequence of isolation from the faeces.

Measure for the CR

We compared two possible objective ways of assessment of a measure for the CR with the purpose of choosing the most economical one.
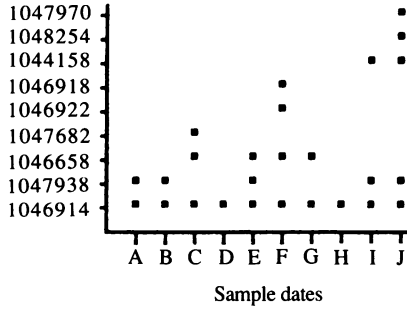
The first approach is presented by the slope of 'the best fitting line' ($y = ax + b$) calculated in the biotyping diagram. This line is calculated through the highest values of the y-axis representing the last isolated (new) biotype, not found in previous samples, on the sequential sample dates. The higher the number of newly acquired biotypes, the steeper the slope of this line. We also calculated the correlation coefficient of this line.

A second approach to find a measure for the CR was to calculate the mean number of different biotypes in stepwise increasing numbers of subsequent faecal samples of a volunteer. The lowest number of faecal samples that provided a statistically reliable mean was determined in the present analysis. The method requiring the smallest number of faecal samples for biotyping could then be used in patient care. We estimated the lowest number of faecal samples in the second approach by applying the standard error of the mean (S.E.M.) of stepwise adding the data derived from subsequent samples. If adding data of additional samples did not alter the standard error of the mean significantly, it was decided that the minimal number of samples had been reached.

RESULTS

The mean number of faecal samples investigated per volunteer was 13, range 10–15. The development of the patterns of the number of different biotypes of two volunteers with time are presented as an example in two diagrams (Figs. 1 *a* and 1 *b*). These examples are given because remarkable differences appeared to exist between these two volunteers. Volunteer 5 for example had a few different

(a)   Biotype

1047970
1048254
1044158
1046918
1046922
1047682
1046658
1047938
1046914

A B C D E F G H I J

Sample dates

(b)   Biotype

588591
689663
591921
591317
985577
723377
854445
723341
661941
657853
67985
6408
76032
526781
127744
592277
592305
541109
526769
655781
657829
68029
590267
592271
524709
68027
657845
657823
524735
592317
854461
592309
723391
886271
526757
985535
723381
656895
657855
526783
886207
526767
592315
723389
558591
526773
592319

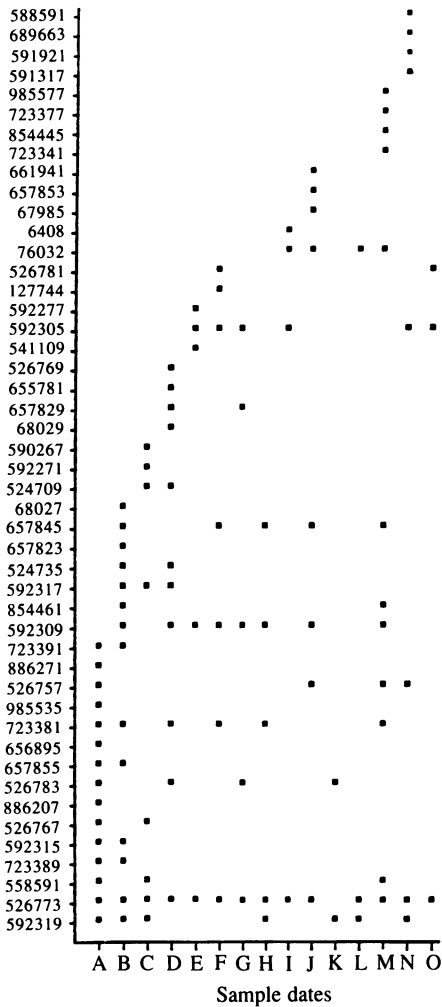A B C D E F G H I J K L M N O

Sample dates

Fig. 1 (a) Biotyping diagram of volunteer 5. (b) Biotyping diagram of volunteer 8.

Table 1. *Mean number of different biotypes, range and SD determined in the faecal samples of nine healthy volunteers. The slope, the intercept and the correlation coefficients of the 'best fitting lines' calculated from the biotyping diagrams of the healthy volunteers are given*

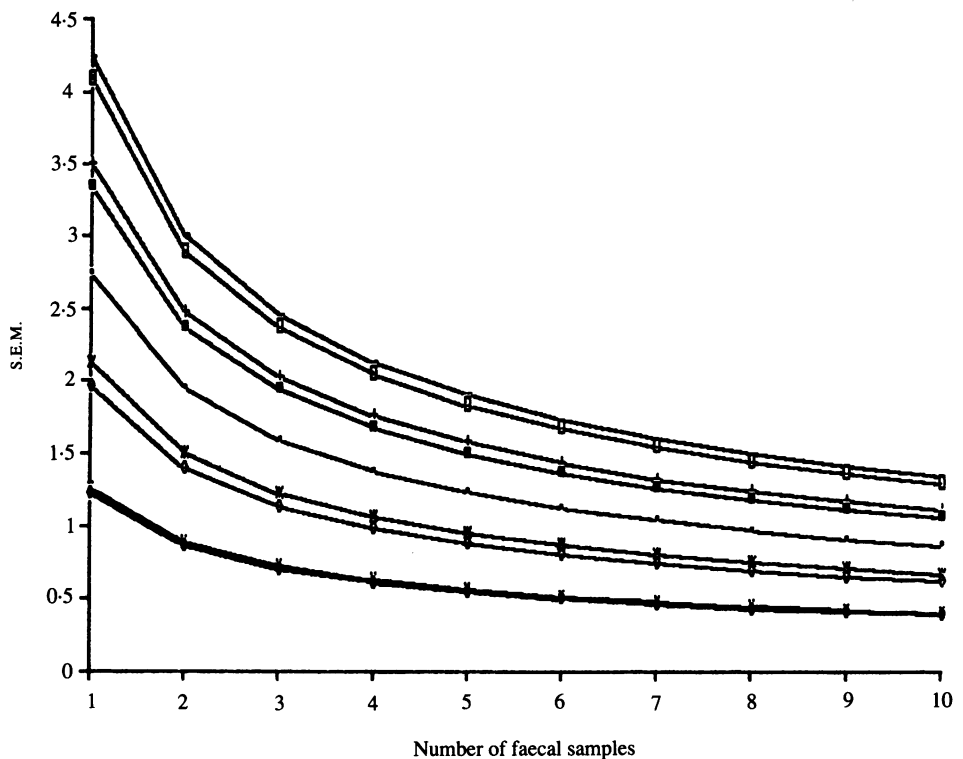| Volunteers...	1	2	3	4	5	6	7	8	9
Mean number of biotypes	5·0	4·9	6·1	3·0	2·6	5·8	3·6	7·3	6·7
Range of the number of biotypes	1–9	2–15	2–9	1–7	1–5	2–14	2–6	2–15	3–13
S.D.	2·74	3·50	2·12	1·96	1·26	4·24	1·22	4·08	3·34
Slope	1·9	1·0	2·3	1·3	0·6	1·1	1·1	1·2	6·7
Intercept	3	2	5	0	1	17	3	20	13

Fig. 2. s.e.m. (number of different biotypes) as a function of the number of faecal samples of nine healthy volunteers: ━ , volunteer 1; +, 2; *, 3; -⊖- , 4; ✳ , 5; ━ , 6; ━ , 7; ⊞ , 8; ━ , 9.

biotypes per faecal sample, mean 2·5, and acquired just a few new ones during the study period (see Fig. 1*a* and Table 1). Volunteer 8, on the other hand, had many different biotypes per faecal sample at the beginning of the study, mean 7·3. In the faeces of this volunteer many new biotypes were isolated in subsequent samples (see Fig. 1*b* and Table 1).

The variation in the number of different biotypes isolated from the faecal samples of these nine volunteers ranged between 1 and 15; the variation in the

mean number of different biotypes per faecal sample was 2·6–7·3. In the mean number of biotypes inter-individual variations of five biotypes per faecal sample were normal. The mean number of biotypes per sample and the range for each volunteer are presented in Table 1.

The correlation coefficients of the calculated 'best fitting lines' through the highest number of new biotypes in subsequent samples ranged between 0·46 and 0·98 with a mean correlation coefficient of 0·68 (S.D. 0·19). The slopes of these lines varied between 1·0 and 6·7, whereas the intercept ranged from 0 to 20.

We calculated the standard error of the mean (S.E.M.) number of different biotypes per faecal sample for each volunteer separately, for increasing numbers of faecal samples (see Fig. 2). Including four faecal samples instead of one for determination of a reliable mean number of different biotypes per faecal sample yielded a considerable gain of 50% in accuracy of determination of that mean number of different biotypes. Including a fifth faecal sample yielded only an extra 5% in accuracy, and adding more samples even less (see Fig. 2).

DISCUSSION

The results of our study show considerable differences in the mean number of biotypes of Enterobacteriaceae excreted per faecal sample by healthy volunteers. In addition, there are also clear differences in the number of new biotypes found in the faeces of these volunteers. These inter-individual differences appeared constant and could be expressed adequately in the mean number of biotypes per sample. These differences may bear a relationship to the quality of the individual CR of the digestive tract of the volunteers.

Using the computer diagrams it is easy to identify inter-individual differences. Searching for an objective, reliable measure for the differences in the number of different biotypes isolated from subsequent faecal samples of each individual, we tried to calculate the 'best fitting line' through the highest values of the y-axis representing the last isolated biotype codes belonging to the sequential sample dates. The value of the correlation coefficients of the 'best fitting line' calculated for each volunteer separately varied between 0·46 (volunteer 6) and 0·98 (volunteer 3) with a mean of 0·68 (see Table 1). The correlation coefficient was sufficiently high ($\geqslant 0·7$) only in 4/9 persons. Therefore, it was decided that this approach, which often requires more than the number of samples used for biotyping in the present study, is not suitable for determining the CR reliably for every individual.

A better and more economic method for objective assessment of a qualitative measure of the CR could be determination of the mean number of different biotypes excreted per faecal sample by an individual. Examination of data of the nine volunteers shows that the number of biotypes per faecal sample per individual does not show a pattern dependent on time. The error in calculating the mean number of biotypes obviously decreases the more samples are involved in the calculation. However, after an initial gain in accuracy of 50% in determination of the mean number of different biotypes when four instead of one faecal samples are involved in the calculation, the increase in accuracy rapidly decreases to only 5% when a fifth faecal sample is included. This relatively small extra increase does

not justify the biotyping of a fifth sample considering the time and the costs involved in processing an extra sample.

To conclude, reliable assessment of the mean number of biotypes as measure of CR can apparently be achieved by examination and biotyping of four faecal samples. Further research is required to prove the validity of this measure of CR in human volunteers. This will be done by performing experimental oral contamination with an Enterobacteriaceae species concomitantly with the presently reported biotyping method. A study in which these two measures are compared, is in progress.

REFERENCES

1. Hentges DJ, Que JU, Casey SW, Stein AJ. The influence of streptomycin on colonization resistance in mice. Microecol Ther 1984; **14**: 53–62.
2. Hofstra W, Welling GW, Waaij D van der. A comparative study of the effect of oral treatment with augmentin, amoxycillin and bacampicillin in the faecal flora in mice. Zentralbl Bakteriol Mikrobiol Hyg [A] 1988; **269**: 78–85.
3. Waaij D van der. The digestive tract in immunocompromised patients: importance of maintaining its resistance to colonisation, especially in hospital inpatients and those taking antibiotics. In: Sabath LD, ed. Action of antibiotics in patients. Bern: Hans Hubert Publishers, 1982: 104–18.
4. Waaij D van der, Speltie TM, Vossen JM. Biotyping of Enterobacteriaceae as a test for the evaluation of isolation systems. J Hyg 1972; **70**: 639–50.
5. Waaij D van der, Speltie TM, Guinee PAM, Agterberg C. Serotyping and biotyping of 160 *Escherichia coli* strains: comparative study. J Clin Microbiol 1975; **1**: 237–8.
6. Waaij D van der, Tieleman-Speltie TM, De Roeck-Houben AMJ. Infection by and distribution of biotypes of Enterobacteriaceae species in leukemic patients treated under ward conditions and in units for protective isolation in seven hospitals in Europe. Infection 1977; **3**: 188–94.
7. Waaij D van der, Berghuis-de Vries JM. Lekkerkerk-van der Wees JEC. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg 1971; **69**: 405–11.
8. Waaij D van der, Berghuis JM. Determination of the colonization resistance of the digestive tract of individual mice. J Hyg 1974; **72**: 379–87.
9. Hahn DM, Schimpff SC, Fortner CL, Smyth AC, Young VM, Wiernik PH. Infection in acute leukemia patients receiving oral nonabsorbable antibiotics. Antimicrob Agents Chemother 1978; **13**: 958–64.
10. Waaij D van der, Tieleman-Speltie TM, De Roeck-Houben AMJ. Infection by and distribution of biotypes of Enterobacteriaceae species in leukaemic patients treated under ward conditions and in units for protective isolation in seven hospitals in Europe. Infection 1977; **5**: 188–94.
11. Waaij D van der, Tieleman-Speltie TM, De Roeck-Houben AMJ. Relation between the faecal concentration of various potentially pathogenic microorganisms and infections in individuals (mice) with severely decreased resistance to infection. Antonie van Leeuwenhoek 1978; **44**: 395–405.
12. Tancrède CH, Andremont AO. Bacterial translocation and gramnegative bacteremia in patients with hematological malignancies. J Infect Dis 1985; **152**: 99–103.
13. Schimpff SC, Young VM, Greene WH. Vermeulen GD, Moody MR, Wiernik PH. Origin of infection in acute nonlymphocytic leukemia. Significance of hospital acquisition of potential pathogens. Ann Intern Med 1972; **77**: 707–14.