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DETERMINATION OF COLONISATION RESISTANCE OF THE DIGESTIVE TRACT BY BIOTYPING OF *ENTEROBACTERIACEAE* ISOLATED FROM SUBSEQUENT FAECAL SAMPLES

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SUMMARY

Nine healthy volunteers were studied during six 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 Colonisation Resistance (CR) of an individual. It was found that minimally four faecal samples are required.

We validated measurement of the CR in 10 healthy individuals by determination of the mean number of different biotypes of *Enterobacteriaceae* isolated from four faecal samples per volunteer by comparison with the golden standard for CR. The golden standard for CR is oral contamination with a neomycin resistant *Escherichia coli* (NR-*E. coli*) strain and measurement of the faecal concentration of this strain during 14 days after the contamination. Both measures are significantly correlated ($p<0.05$). The NR-*E. coli* strain could be cultured from faecal samples of 4/10 volunteers as long as 300 days after contamination.

INTRODUCTION

Man and his microflora constitute an intriguing ecosystem. The intestinal bacteria even outnumber the eukaryotic host cells. Yet both man and his microflora succeed in peaceful symbiosis. Not much is known about this ecosystem because its numerous relations are difficult to study.

Now that the direct relations are difficult to approach, perhaps it is possible to deal with the results of the interactions. One such result of the myriad of interactions between the host and his intestinal microflora is the Colonisation Resistance (CR). CR is a property of the indigenous intestinal microflora that controls the growth and therewith the chance of translocation of potentially pathogenic bacteria across the gut wall. CR is accomplished by means of the action of antimicrobial metabolites (*Walker*, 1990). Other factors that influence colonisation of bacteria are ability to adhere to the gut wall and the

length of the lag phase of the bacteria (*Freter*, 1983, 1986). Competition for niches and nutrients e.g. has also been assigned a role in this prevention of colonisation by foreign bacteria. The role of the indigenous mainly anaerobic flora in the maintenance of the CR for potentially pathogenic bacteria was shown indirectly in studies in which antibiotics were administered. Antibiotics that killed many anaerobic bacteria decreased the CR, whereas antibiotics that left the anaerobic part of the microflora as it was, did not affect the CR (*Hentges* et al., 1984; *Hofstra* et al., 1988; *van der Waaij* et al., 1972a; *van der Waaij*, 1982).

The stronger the suppression of ingested bacteria by the anaerobic flora, i.e. the stronger the CR, the less different potentially pathogenic bacteria are apparently able to colonise the gut. *Enterobacteriaceae* species - particularly *E. coli* biotypes - in general form an important part of the facultatively anaerobic (aerobic) flora of the digestive tract. The quality of the CR and therewith its protective capacity may vary between individuals of the same species (*van der Waaij and Heidt*, 1990).

The digestive tracts of individuals with an impairment of their CR, e.g. due to destruction of large parts of the anaerobic flora by antibiotic treatment, are likely to become colonised in high concentrations by potentially pathogenic bacteria (*Hahn* et al., 1978; *van der Waaij* et al., 1977, 1978). Dominant clones of *Enterobacteriaceae* - those that grow out to high numbers - represent the strains which are most likely to translocate to lymphatic organs and the liver (*Tancrede*, 1985). In immunocompromised patients translocation of *Enterobacteriaceae* may result in a life-threatening septicaemia (*van der Waaij* et al., 1977, 1978; *Schimpff* et al., 1972). The concept of CR and translocation of potentially pathogenic bacteria in case of impairment of the CR form the rationale behind selective decontamination of the digestive tract (SDD) in immunocompromised patients (*van der Waaij*, 1992).

CR was first studied in the seventies in mice. In mice, CR is defined as the resistance of the digestive tract against colonisation by orally ingested potentially pathogenic bacteria (*van der Waaij* et al., 1971). In mice CR has been defined as the $\log^{10}$ of the oral bacterial dose followed by a persistent "take" in 50% of the contaminated animals (*van der Waaij* et al., 1971). For an individual mouse the CR can be directly expressed as the $\log^{10}$ concentration of a specific potentially pathogenic bacterial species found in the faeces two weeks after contamination. Measurement of CR can also be accomplished by quantitative biotyping of one or more *Enterobacteriaceae* strains isolated from faeces collected at three of four daily intervals. This was considered a useful method because of the finding that, after oral administration of a single dose of an *Escherichia coli* strain to mice, the population density of these bacteria in the intestinal tract (faeces) varied inversely with CR (*van der Waaij* and *Berghuis*, 1974).

We have analysed the data of a comprehensive biotyping study in healthy volunteers performed in the seventies. 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 (*van der Waaij* et al., 1972b, 1975]. 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 investigated how many faecal samples should be studied minimally in order to obtain a reliable value for the mean number of

different biotypes of *Enterobacteriaceae* as a measure for CR.

Furthermore, this method of determining the CR was validated in healthy human volunteers by comparison with a golden standard for CR: Oral contamination with an *E. coli* strain and measuring its faecal concentration two weeks after administration. Ten healthy volunteers were given orally a NR-*E. coli* strain. Thereafter the mean faecal concentration of this strain was assessed from day 1 to day 14. These concentrations were compared with the mean number of different biotypes of *Enterobacteriaceae* in the faeces in four faecal samples obtained from each volunteer within one week prior to the oral contamination (*Apperloo-Renkema* et al., 1990).

SUBJECTS, MATERIALS AND METHODS

Natural variation in biotypes of *Enterobacteriaceae* and minimal number of faecal samples to be studied

Subjects

Nine healthy volunteers, seven male and two female, aged 22-51 years, participated in the study. None of them had taken antibiotics for eight weeks 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 six 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 colonies was cultured per sample. Details of the biotyping technique have been described previously (*van der Waaij* et al., 1972b).

Determination of the minimal number of faecal samples

We estimated the lowest number of faecal samples by applying the standard error of the mean (SEM) 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 (*Apperloo-Renkema* et al., 1990).

Statistical analysis

Statistical software has been developed for data storage and processing of the results of the biotyping experiment (*Apperloo-Renkema* et al., 1990).

Oral contamination and biotyping

Volunteers and sampling

Ten healthy volunteers, seven males and three females, aged 22-44, entered this study after having given written informed consent. The experiment was approved of by the Medical Ethical Committee of the University Hospital Groningen.

Before oral contamination, four faecal samples were collected per volunteer within one week. After oral contamination with an *E. coli* strain, faecal samples were collected daily, whenever faeces were produced. After three months

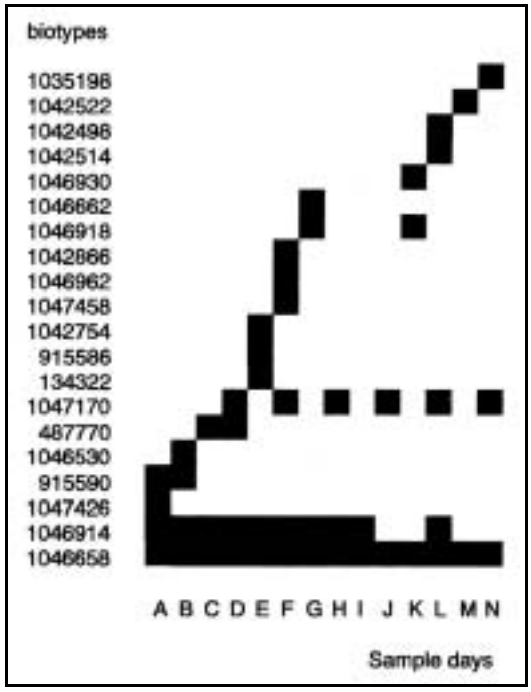

Figure 1: Biotyping diagram of a healthy individual. On the X-axis the sample days are shown, on the Y-axis different biotypes of *Enterobacteriaceae* are given.

the frequency of faecal sampling diminished to once per month. In the period of one month before until three months after the oral contamination, no volunteer had suffered or did suffer from a gastrointestinal disease or had taken antibiotics.

Oral contaminating strain

The *E. coli* strain used for oral contamination was identified with the API-20E system (Analytab Products Inc., Montalieu Vercieu, France). The biotype of the strain was 1144512. The strain was resistant to neomycin (minimal inhibitory concentration >250 mg/l). The strain will be referred to as NR-*E. coli*. For oral contamination the volunteers ingested 10 ml chocolate milk to which 1 ml of an overnight culture at 37°C (approximately 10^9 bacteria/ml) of the NR-*E. coli* strain had been added.

Determination of the faecal concentration of the NR-E. coli strain.

Faeces were suspended 1:9 (w/v) in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) and subsequently diluted 1:9 (v/v) in BHI. After overnight incubation at 37°C the suspensions were subcultured on MacConkey agar (Merck, Darmstadt, FRG) to which neomycin had been added (250 mg/l). The concentrations were determined by standard dilution methods for both the NR-*E. coli* strain the endogenous strains of *Enterobacteriaceae*.

Measurement of CR by comprehensive biotyping of Enterobacteriaceae

CR was defined as the reciprocal value of the mean number of different biotypes of *Enterobacteriaceae* isolated from four faecal samples obtained within one to two weeks (*Apperloo-Renkema* et al., 1990). Details of the

Table 1: Results of the first study on the natural variation of the number of biotypes of *Enterobacteriaceae* found in faecal samples of nine healthy individuals

	healthy individuals								
	1	2	3	4	5	6	7	8	9
Number of faecal samples:	13	14	15	13	10	12	14	15	14
Mean number of different biotypes of *Enterobacteriaceae*:	5.0	4.9	6.1	3.0	2.6	5.8	3.6	7.3	6.7
Range of different biotypes of *Enterobacteriaceae*:	1-9	2-15	2-9	1-7	1-5	2-14	2-6	2-15	3-13

biotyping technique have been described previously (*van der Waaij* et al., 1977). The strains were stored at -20°C until use for specific antibody titration.

Measurement of CR by oral contamination

CR can be expressed directly as the $\log^{10}$ concentration of a specific potentially pathogenic bacterial species found in the faeces two weeks after contamination. Because of the normal fluctuations in the faecal concentration of *Enterobacteriaceae* we assessed the mean faecal concentration of this strain daily on day one until day 14 after oral contamination instead of the faecal concentration on day 14 only.

RESULTS

Natural variation in biotypes of *Enterobacteriaceae* and minimal number of faecal samples to be studied

In the first study the mean number of faecal samples investigated per volunteer was 13, range 10-15. The development of the pattern of the number of different biotypes of a volunteer with time is presented as an example in a diagram (Figure l). 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 (Table 1). In the mean number of biotypes inter-individual variations of five biotypes per faecal sample were found. There was a random variation of number of different biotypes with time.

We calculated the standard error of the mean (SEM) number of different biotypes per faecal sample for each volunteer separately, for increasing numbers of faecal samples. 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.

Oral contamination and biotyping

The strains of *Enterobacteriaceae* isolated from the faecal samples of the volunteers collected in the week before the oral contamination were sensitive to neomycin in contrast to the strain used for oral experimental contamination. The oral intake of the chocolate milk with the NR-*E. coli* strain was well tolerated as none of the volunteers suffered from any clinical symptoms. The

Table 2: Results of the study on oral contamination and biotyping in 10 healthy individuals

	healthy individuals									
	1	2	3	4	5	6	7	8	9	10
Mean number of different biotypes of faecal *Enterobacteriaceae* before oral contamination:	2.00	1.25	1.25	2.25	2.00	1.25	2.25	2.00	2.25	1.25
Mean faecal concentration of NR-*E. coli*, day 1-14 after oral contamination*:	4.4	1.3	3.3	4.3	4.0	2.2	4.4	3.8	4.8	3.7
Mean concentration of faecal Enterobacteriaceae before oral contamination:	3.4	3.0	4.4	4.1	4.6	4.5	3.5	3.0	3.5	2.4

*: ^{10}log/g faeces

strain was cleared from the intestines within 14 days in one case. After one year four volunteers were still colonised with the NR-*E. coli* strain.

The mean number of different biotypes isolated from the faecal samples collected prior to the oral contamination ranged between 1.25 and 2.25 different biotypes between the volunteers (Table 2). The mean concentration of the NR-*E. coli* strain measured from day 1 to 14 correlated significantly with the mean number of different biotypes of *Enterobacteriaceae* isolated from the four faecal samples of the volunteers collected before the oral contamination ($r = 0.80$, $p<0.05$).

The mean concentration of *Enterobacteriaceae* in the faecal samples before the oral contamination ranged between 2.4 and 4.6 ($\log^{10}$/g faeces). No correlation was found between the mean concentration of *Enterobacteriaceae* in the faecal samples before the oral contamination and the mean concentration of the oral contaminating strain measured from day 1 to 14 ($r = -0.01$).

DISCUSSION

The results of our study show considerable differences in the mean number of biotypes of *Enterobacteriaceae* excreted by healthy volunteers per faecal sample. 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; this possible relationship was subject of the second study presented here.

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. Reliable assessment of the mean number of biotypes as measure of CR can apparently be achieved by ex-

amination and biotyping of four faecal samples.

In 10 healthy volunteers CR was measured in two ways. The first, the "golden standard" worked out in mice, is the faecal concentration of an oral contaminating strain measured after 14 days, and the second was a measure applicable in hospitalised patients for studies of the effect of antibiotic treatment on the protective indigenous microflora (*van der Waaij* et al., 1977). Since the concentration of the oral contaminant in faeces was not constant but fluctuated, the mean concentration on days 1 to 14 was used instead of those on day 14. Both measures for CR were significantly correlated ($p<0.05$). Therefore, we decided to use biotyping of *Enterobacteriaceae* isolated from four faecal samples obtained within one week as a measure for CR instead of the ethically less acceptable method of oral contamination. In the first study, the mean number of different biotypes of *Enterobacteriaceae* in the healthy individuals was found to be higher than in the second study. This difference is mainly accounted for by the difference in biotyping techniques that were applied in the respective studies. The elder method focussed on as many differences in outcome of biochemical reactions as possible (*van der Waaij* et al., 1972b), whereas the latter (API system) targeted at as many different strains of *Enterobacteriaceae* as possible. The first approach yielded more different "biotypes" than the second.

In this study, we found no correlation between concentrations of endogenous *E. coli* strains and the concentration of the NR-*E. coli* strain on day 14 after contamination. Higher concentrations of endogenous *Enterobacteriaceae* are found sometimes after broad-spectrum antibiotic therapy and are then indicative for a disturbed intestinal ecosystem and a lowered CR. But in the normal situation the concentration of endogenous *Enterobacteriaceae* cannot be used as such an indicator.

One might hypothesise that the higher the CR, the sooner a newly ingested bacterium from the environment will be eliminated from the gastrointestinal tract. However, the speed of clearance depends not only on the strain but also on the number of bacteria ingested. Ingestion of 10^9 bacteria of a strain by the volunteers in our study may rarely occur in normal life. Indeed, in several subjects the elimination of the NR-*E. coli* strain lasted quite long. Clearance of laboratory strains (*Kaijser*, 1983) or *Pseudomonas aeruginosa* (*Buck*, 1969) occurs within a few weeks to a few months.

This measurement of CR provides us with a tool for the study of one aspect of the ecosystem constituted by man and his intestinal microflora. E.g. it is possible now to study differences in CR between different animal species (*van der Waaij* and *van der Waaij*, 1990). Moreover the relationship between the CR and development of wasting disease in mice was studied this way (*van der Waaij* and *Heidt*, 1990). A possible relation between CR and activity of Systemic Lupus Erythematosus (SLE) in man was also subject of study and revealed slight differences between patients with active and inactive SLE versus healthy individuals (*Apperloo-Renkema*, unpublished results).

To conclude: We assessed natural variation in biotypes of *Enterobacteriaceae* in faeces from healthy individuals. The minimal number of faecal samples was assessed that should be taken for comprehensive biotyping in order to determine reliably the mean number of different biotypes representative for the Colonisation Resistance (CR) of an individual. It was found that minimally four faecal samples are required.

We validated measurement of the CR

in 10 healthy individuals by determination of the mean number of different biotypes of *Enterobacteriaceae* isolated from four faecal samples per volunteer by comparison with the golden standard for CR. The golden standard for CR is oral contamination with a neomycin resistant *Escherichia coli* (NR-*E. coli*) strain and measurement of the faecal concentration of this strain during 14 days after the contamination. Both measures are significantly correlated ($p<0.05$). The NR-*E. coli* strain could be cultured from faecal samples of 4/10 volunteers as long as 300 days after contamination.

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