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The colonization resistance of the digestive tract in different animal species and in man; a comparative study

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SUMMARY

The present study has attempted to determine the colonization resistance (CR) of the digestive tract by biotyping Enterobacteriaceae in four faecal samples per subject of five different animal species as well as man. The results indicate that the degree of bacterial contamination with Enterobacteriaceae from the environment may strongly influence the outcome. Both conventionally living chicken and man, showed a much wider range of the 'confidence limits of the mean' of the mean number of biotypes per faecal sample between individual subjects, than was found between subjects maintained under laboratory circumstances. Yet there appeared a statistically significant difference in CR between some of the animal species as a group. Man did not differ from monkeys, however, both differed from the rodents species studied. Monkeys differed also from dogs and the latter from rodents. It is concluded that the CR measured by determining the mean number of biotypes of Enterobacteriaceae can only be used for accurate comparison of the CR between subjects, if the 'bacteriological environment' is known; i.e. the sources of contamination.

INTRODUCTION

In the last two decades evidence has accumulated that the indigenous intestinal microflora plays a key role in the control of colonization by newly-ingested bacteria. This property is called colonization resistance (CR) of the digestive tract [1, 2]. The composition of the indigenous microflora is greatly determined by its host and cooperates closely with the host [3]. Because the intestinal microflora differs clearly between animal species, it was decided to investigate whether this implies a different CR. A comparative study of CR was therefore performed in man and in different animal species.

In the present study in each animal species, the CR was determined in a number of individuals by comprehensive biotyping of Enterobacteriaceae. Previously, it has been shown in laboratory mice that there is a relationship between the CR measured by experimental oral contamination and the mean number of biotypes per faecal sample [4]. This approach has been described previously in mice [5]. For the determination of the CR by biotyping at least four faecal samples per individual animal/man must be investigated [6]. It is to be expected that, with this method for determining the CR, individuals maintained in a conventional

environment with many different Enterobacteriaceae often in large quantities, might ingest and excrete a higher mean number of biotypes in their faeces than individuals maintained under strict hygienic specific pathogen free (SPF) conditions. Consequently, inter-individual differences in CR might be more clearly expressed in conventionally maintained subjects.

Animal species investigated were SPF mice, guinea-pigs, laboratory-maintained monkeys and dogs, and conventionally raised chicken and man. Man and monkeys, being primates, might have a CR of comparable quality. Mice and guinea-pigs being rodents, might also share CR values. The dog might differ from the two but also from the chicken.

MATERIAL AND METHODS

Human volunteers and animals

These comprised 9 young adult (25–40 years) healthy human volunteers (males and females), 7 healthy young adult monkeys (*M. mulatta*) (4 males and 3 females derived from the Primate Center of TNO, Rijswijk, The Netherlands). Twenty-one healthy young adult Beagle dogs (7 males and 14 females born and raised until the experiment in the (clean conventional) Animal Breeding Unit of TNO, Zeist, The Netherlands), 8 3–4 months old specific pathogen free (SPF) English Short-hair albino guinea-pigs (4 males and 4 females), 36 healthy SPF mice (20 ND2/Rij and 16 C3H/Rij mice; 9 males and 27 female animals), and finally 7 conventional chickens (Leghorn, females only).

Maintenance circumstances of the various animal species

Circumstances under which the animals were maintained, differed. Mice were held five to a polycarbonate transparent clean cage, while guinea-pigs and monkeys were kept in separate wire cages. The dogs had an outdoor kennel with a concrete floor which was daily hosed with water, they shared this space with 3–5 mates. The seven chickens had an outdoor run with a soil floor and shared this space. Besides cage mates (in mice) and caretakers, food may have been a source of new Enterobacteriaceae biotypes for oral ingestion. All animals except the chicken were fed with (non-autoclaved) pelleted laboratory food (Hope Farm, Woerden, The Netherlands). Monkeys received in addition daily some disinfected fresh fruit, while the chicken were fed with conventional mixed grain and salad. The way dogs and monkeys were held is generally referred to as 'clean conventional', because their housing circumstances permit daily cleaning and because their pelleted food has a very low bacterial count of less than ten bacteria per gram.

The human volunteers lived with their families (except for working hours) and took the conventional Western European type of diet.

Faecal sampling

Fresh faeces were sampled two to three times a week for subsequent comprehensive biotyping. Each individual was a minimum 2 weeks on study with a median of 4 weeks: some of the human volunteers, and some monkeys and dogs, were sampled for 6 subsequent weeks.

Because of the laboriousness and the high costs of biotyping, the study was spread over 5 years. The study in the monkeys, the dogs, the chicken and the mice took 3 years in total. In each of these animal species, one or two individuals were sampled at the time. Man and guinea-pigs were sampled within a shorter period of between 6 months and a year. After sampling the faeces were processed immediately in the laboratory.

Comprehensive biotyping of Enterobacteriaceae for determining CR

This technique has been described previously in great detail [5]. Briefly, fresh faeces were inoculated directly onto McConkey agar (Difco) and Endo agar (Difco) as well as following preincubation in Brain Heart Infusion broth (Difco) for 24 h. After overnight incubation at 37 °C, as many different looking colonies (minimally 20) were picked for pure culture and subsequent typing with API 20E strips (Analytab Products Inc. New York, Lyon, France). During picking of the colonies for typing, each colony received a sequential number. It could then be calculated to what extent the number of colonies picked represented the number of different biotypes in the sample. If there was evidence that the number of colonies was insufficient, as many additional colonies were picked from the plates as required.

Statistical analysis

To estimate to what extent the mean number of biotypes is normally distributed per animal species, the 'rankits to the rescue' [7] were determined of these data. Because the rankit procedure indicated a normal distribution of the mean number of biotypes in each animal species, the double sided t-test was applied to determine the confidence limits of the means. Groups were compared where this was statistically permitted; i.e. the standard deviation of the mean (S.D.) of both groups did not differ significantly. This is a prerequisite for the t-test. For comparison of the SD's of the groups, the double sided F-test was used [7].

RESULTS

The results of the present study are presented in Table 1. The mean number of biotypes found in the faeces per animal species and the standard deviation of this mean, differed significantly between the animal species (Fig. 1). For comparison of these means it is important to know that the 'rankits to the rescue' test has indicated that the mean number of different biotypes of Enterobacteriaceae/faecal sample was normally distributed. With this information, the confidence limits of the mean could be calculated and are depicted in Fig. 1. The results in this figure show two remarkable phenomena:

(i) The mean number of biotypes per faecal sample differed between individuals of each animal species; greater in conventionally raised species (man and chicken) than in those raised under 'clean conventional' conditions or SPF conditions.

(ii) The mean number of biotypes (mean CR) differed between animal species as far as comparison is permitted by the F-test (Table 2) at different levels of significance. Man and monkeys (conventional) differed in the F-test at a 1% level and could therefore be compared. Comparison was also permitted by the F-test

Table 1. *Mean number of different biotypes of Enterobacteriaceae and standard deviation (S.D.) and standard error of the mean (S.E.M.) in six different animal species including man*

Animal species	No.	Mean no. of biotypes	S.D.	S.E.M.	Breeding conditions
Mouse	29	1·55	0·28	1·51	SPF
Guinea-pig	8	1·81	0·24	0·68	SPF
Dog	21	2·76	0·82	3·76	clean conventional
Chicken	7	3·42	1·01	2·67	conventional
Monkey	7	4·81	0·44	1·16	clean conventional
Man	9	5·00	1·66	4·98	conventional

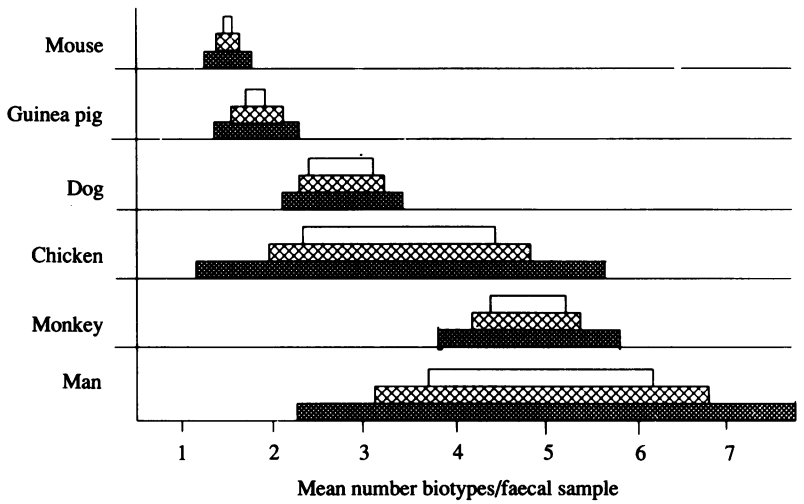

Fig. 1. Confidence limits, at three different levels, of the mean number of biotypes of Enterobacteriaceae isolated from four faecal samples from a (different) number of subjects per animal species studied and man. ▨, 99·9%; ▩, 99·0%; □, 95·0%.

Table 2. *Outcome of the F-test for the difference between standard deviations of the mean number of biotypes of Enterobacteriaceae/faecal sample*

Groups compared	Significance	Groups compared	Significance
Man/mouse	1%	Monkey/mouse	N.S.
Man/guinea-pig	1%	Monkey/guinea-pig	N.S.
Man/dog	1%	Monkey/dog	5%
Man/chicken	N.S.	Monkey/chicken	5%
Man/monkey	1%	Chicken/dog	N.S.
Dog/mouse	1%	Chicken/guinea-pig	1%
Dog/guinea-pig	1%	Chicken/mouse	N.S.

between man and most animal species except chicken as well as between monkeys and dogs and between chicken and mice (Table 2).

The two primate species appeared not to differ significantly from each other. Man and monkeys differed significantly from both rodent species. Furthermore, monkeys (clean conventional) differed highly significantly from dogs, chicken and both rodent species ($P < 0·001$). Dogs differed significantly ($P < 0·01$) from the

rodent species. As shown in Table 1 and Fig. 1, the lowest mean number of biotypes of Enterobacteriaceae per sample was found in the rodents.

DISCUSSION

The results of the present study indicate that the highest mean value of the CR (inverse relation to mean number of biotypes) in relation to their 'exposure to contamination' (the number and different biotypes of Enterobacteriaceae species circulating in their environment) was seen in mice. The lowest average value of the CR was observed in man and monkeys. The maintenance conditions may obviously have determined their exposure to biotypes of Enterobacteriaceae. This could explain why man and chicken, both raised and maintained conventionally, showed the widest range in the confidence limits of the mean numbers of biotypes found per faecal sample. Another obvious consequence of the present findings is that biotyping of Enterobacteriaceae species in faecal samples can only be used for determining the CR if the bacteriological environment of the species examined is comparable; i.e. if their exposure to contamination with Gram-negatives is comparable. This may only occur in rooms for experimental animals and not in individuals in conventional free-living situations such as existed in man and chicken.

If regarded as a group, man and monkeys as well as the chicken did not have a significantly different mean number of biotypes. The human volunteers lived at home with their families except for working hours. The five volunteers who had daily contact with animals all had a mean of more than five biotypes per faecal sample; the volunteer who had daily contact with monkeys had the seven biotypes, the highest mean number. Contact with animals and in particular with monkeys may have implied a greater exposure to more different biotypes and in higher quantities than in laboratory workers. It has recently been shown that direct transmission may occur between animals (chicken) and their caretakers [8]. The chicken lived conventionally; i.e. outdoors in a chicken run. They may therefore, have eaten insects which it is known may be colonized by Enterobacteriaceae [9]. The data obtained in man and chicken may consequently provide insight into what could be a 'normal natural situation'.

The monkeys were maintained in separate cages in an animal room. In this respect, they were comparable with the guinea-pigs and mice. Comparison of the mean values presented at different confidence levels in Fig. 1 is not permitted by the F-test. Whether this is species specific or due to a difference in exposure is still an open question. The monkeys had not been raised under SPF conditions but 'clean conventionally'.

Because highly significant differences were found between the rodent species and both primate species as well as dogs, it is likely that, in addition to breeding and maintenance circumstances, species really differ in CR. The CR differences may form the decisive factor in the determination of the mean number of biotypes per faecal sample. The hygienic quality of the environment may be a factor of subsequent importance. This assumption is to some extent supported by the fact that there is a S-shaped relationship between the log oral infection dose and the log faecal concentration of the Gram-negative contaminant after a standard

interval of 4 days [4]. Secondly, in a total of 27 food samples we found a linear correlation ($r = 0{\cdot}84$) between the log concentration of bacteria in the food pellets of mice, dogs and monkeys and the number of different biotypes of Enterobacteriaceae species among those bacteria (data not shown).

Our findings regarding the influence of maintenance conditions lead to the following tentative hypothesis: if an animal species is maintained under increasingly unhygienic circumstances and is therefore exposed to an increasing number of different biotypes in high quantities in their environment, the mean number of biotypes per faecal sample may increase. Unhygienic environmental conditions may, because of the S-shaped correlation between oral contamination dose and the mean number of biotypes per sample, enhance the expression of the strength of the CR and therewith inter-individual differences. Inter-individual differences within one animal species therefore, reflect the relation between the quality of an individual's CR and the contamination level. The assumption that a greater exposure to Enterobacteriaceae may result in a higher mean and range of biotypes per faecal sample in a particular animal species is plausible, since this was observed in a previously published controlled experiment in mice [10]. During the experimental period, 36 conventional mice were maintained inside a germ-free isolator with, in total, 12 different biotypes of Enterobacteriaceae. These 12 biotypes included both the biotypes excreted by the animals in their faeces (cross-contamination) and biotypes isolated from a sample of the food pellets with which the animals were locked into the isolator. Cages, bedding material, drinking bottles and drinking water had been autoclaved before they were introduced into the sterile isolator. The mean number of biotypes found in these individually sampled (on the average eight samples/animal) mice was 2·08 (S.D. = 0·77) and 1·55 in the present study. Because the mice involved in the present study were maintained in an animal room and therefore exposed to an unknown number of biotypes, no information on this point is available. However, in their food pellets the number of biotypes may have been three at the most, according to the culture results of routine samples taken quarterly. In most (larger) animals and man the contamination level in the environment can not be determined with sufficient accuracy. This implies that we have to rely on approaches as indicated in the present study. Insight by a study of this kind is considered necessary because it has important implications for the use of, for example, rodents raised and maintained under SPF conditions and with a significantly higher CR as a model for conventionally living man.

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