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Phenotypic Diversity and Stability of the Intestinal Coliform Flora in Piglets During the First 3 Months of Age

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The intestinal coliform bacterial populations in six piglets and their sows from two litters in the same stable were studied by the aid of an automated system for biochemical fingerprinting (The PhenePlate, or PhP system). Faecal samples from sows were collected once a week until weaning, and from piglets once a week during their first 3 mth of life. Altogether 1248 coliform isolates from 52 faecal samples were studied. The phenotypical diversity of the coliform bacteria in each faecal sample was measured as Simpson's diversity index (D_i), and the similarities between the bacteria in different samples were measured as population similarity coefficients (S_p). The D_i of the sows was initially high, but decreased in one sow during an outbreak of diarrhoea which occurred during week 3 of the study. The D_i of the piglets varied between samples, and showed no tendency to increase with age. Two piglets included in the study died during the diarrhoeal outbreak, and they generally carried intestinal floras showing lower D_i during their first 3 wk of life than those which survived (mean value 0·454 versus 0·744). The coliform population within each piglet was usually quite stable before the diarrhoeal outbreak, but in all piglets the population was drastically changed during and after the outbreak. Piglets within each litter shared similar coliform populations during the first week of life, but piglets from different litters had no coliforms in common. On day 75, the piglets were removed from their sows and mixed with others in different litters, and after that coliform populations of piglets from different sows showed a tendency to become more similar to each other. Samples collected during the diarrhoeal outbreak from different piglets did not contain similar coliform populations which is a strong indication that the aetiological agent of the outbreak was not any coliform bacterium. The present study shows a useful way to investigate intestinal bacteria in both man and animals. The methods are very simple to use, and fully computerised data treatment makes it possible to study large numbers of bacterial isolates.

KEY WORDS—Intestinal bacteria; Coliforms; Piglets; Diversity; Stability.

INTRODUCTION

The indigenous microflora plays an important role in the health and disease of humans and animals.[4,17,18] One function of this microflora is its ability to suppress colonisation of potential pathogens to body surfaces which would otherwise be sensitive to invading microorganisms.[5,23] An adult individual carries an abundant intestinal microflora, and it has been postulated that more than 400 bacterial species are members of the human intestinal flora.[15] This high diversity of the intestinal flora is believed to have a stabilising effect on the intestinal ecosystem,[16] and thus a decrease in microbial diversity may result in intestinal disturbances and increased susceptibility to various diseases.

The intestinal microflora of humans and animal consists of two types of strains: resident and transient.[19] Transient strains are referred to as those which are only occasionally found in the intestine, possibly introduced by food, but which never become members of the permanent flora. Resident strains are such bacteria which become established in the host for a long time. These bacteria may have

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a mechanism to maintain their population size in the intestine, such as adhesive factors.

Evaluation of the diversity of the intestinal flora is assumed to be an adequate approach to understand the functional status of the ecosystem.[2] In practice, measurement of bacterial diversity is a difficult task, since it involves analysis of many bacteria from each intestinal microflora.[1] However, by calculating the diversity index from a representative sample of the intestinal flora, a good estimation of the total phenotypical diversity may be obtained by identifying 20–30 bacterial isolates from each sample.[3]

We have previously used an automated system for biochemical fingerprinting to type bacterial strains belonging to various coliform species (The PhenePlate or PhP system).[7–10,12] The system is based on the evaluation of the kinetics of bacterial metabolism of highly discriminating substrates, performed in microplates. Recently the PhP system has been evaluated for studying the diversity and the stability of coliform bacterial populations in water samples,[13] and the system was found to be highly discriminating and was able to follow the spread of bacterial phenotypes between different water samples. Since the PhP system is also very simple to use, and can be applied to studies involving large numbers of isolates, it is also an excellent tool for studying the contents of intestinal bacterial floras. In the present study we have used this system to study the development of the intestinal coliform floras in six piglets during their first 3 mth of life.

MATERIALS AND METHODS

Piglets studied

In total six piglets (designated P2, P3, P4, R1, R2, R4) from two litters (P and R) and their sows (SP and SR) were included in the study. The piglets were part of a larger investigation on the effects of environmental stress on the growth of rearing piglets. The piglets were fed by their sows, and commercial feed-pellets were added after day 7. They were weaned on day 35, and on day 75 they were mixed with other piglets in different boxes according to their weight. The piglets were normally not given any antimicrobial drugs, although during a diarrhoeal outbreak one of the piglets in the present study (piglet P4) was administered sulphametoxazol-trimethoprim for 3 d. Two piglets initially included in the present study (piglets P3 and R4) died during a diarrhoeal outbreak in their third week of life. After weaning (sample no. 5) the sows were excluded from the sampling. Thus four piglets (P2, P4, R1, R2) were sampled during the whole study.

Faecal samples were taken from the piglets and their sows by inserting 3 cm of a swab into the rectum. The swabs were transported in Cary and Blair transport medium, stored at +4°C, and cultured within 24 h. The first sample from each piglet was taken on the third day after birth, and then once a week for 3 mth.

Bacterial isolates

The faecal samples were cultured on McConkey agar plates. After 24 h of aerobic incubation at 37°C, 24 pure colonies from each sample were randomly picked for biochemical fingerprinting. In total, 52 faecal samples with 24 isolates each were studied, yielding 1248 bacterial isolates altogether. Species identification, when applicable, was performed with the API 20E system (La Balme les Grottes, France).

Biochemical fingerprinting

The PhP-RS plate (BioSys inova, S-113 51 Stockholm, Sweden) consists of 96 well microplates with eight rows of 11 dehydrated reagents each.[14] The reagents include: Cellobiose, sucrose, gentobiose, raffinose, adonitol, sorbose, L-fucose, arbutin, citrate, malonate and ornithine. A growth medium (0·1 per cent Proteose Peptone and 0·01 per cent Bromothymol blue) was dispensed to the wells of the PhP-RS plates. To the first well in each row, which did not contain any reagent, 0·375 ml was added, and to the other 11 wells 0·125 ml. One colony of each isolate to be tested was picked directly from the MacConkey agar plates with the aid of a 1 µl plastic inoculation loop and suspended into the first well of each row of a PhP-RS plate. The plates were left at room temperature for about 1 h. Using a variable multichannel pipette, the bacterial suspensions in the first well of each row were then homogenised by sucking up and blowing out, and 0·025 ml of this suspension was transferred to each one of the other wells in the same row. The inoculated plates were incubated at 35°C for 3 d. The absorbance value (A_{620}) of each well was measured after 16, 40 and 64 h incubation with a microplate reader (Titertek Multiskan, Flow Laboratories) and automatically transferred to a personal computer.

Calculations

The biochemical fingerprints of all isolates were compared pairwise, and the similarity between each

Table 1. Diversity of the faecal coliform flora in six piglets and their sows

Sample no.	Piglet						Sow	
	P2	P4	R1	R2	P3	R4	P	R
1	0·308	0·964	0·736	0·819	0·692	0·000	0·945	0·932
2	0·569	0·822	0·742	NT	0·696	0·650	NT	NT
3†	0·967	NT	0·721	0·797	0·233	†	0·931	0·641
4	0·703	0·424	0·812	0·862	†		0·971	0·946
5‡	0·906	0·409	0·630	0·500			0·775	0·239
6	0·841	0·540	0·859	0·873				
7	NT	NT	0·783	0·696				
8	0·851	0·902	0·703	0·507				
9	0·678	0·755	0·543	0·895				
10§	0·797	0·634	0·808	0·551				
11	0·630	0·909	0·446	0·895				
12	0·652	0·808	0·641	0·808				

NT, not tested.
*Diarrhoeal outbreak.
†Died during the diarrhoeal outbreak.
‡Weaning.
§Mixing.

pair of strains was calculated as the correlation coefficient (r). Isolates showing correlation coefficients to each other higher than the identity level[11] were assigned to the same biochemical phenotype (BPT).

The phenotype diversity of a bacterial population was measured using Simpson's index of diversity (D_i).[6] D_i in the present study depends on the distribution of isolates into different BPTs. It is high (maximum value 1·0) for a population consisting of many different BPTs, and low (minimum value 0·0) if certain BPTs are dominating the population.

The phenotypic similarity between the bacterial populations in two samples was calculated as the population similarity coefficient (S_p).[13] S_p is a measure of the proportion of strains that are identical in two compared populations. It is high (maximum value = 1) when the populations contain the same dominating strains, and it is low (minimum value = 0) when the compared populations consist of different strains. Comparisons of bacterial populations from different samples yield a matrix of S_p coefficients. In order to visualise the relations between certain bacterial populations, the S_p coefficients were clustered according to the UPGMA method,[20] yielding a dendrogram. In the dendrogram, each sample (consisting of 24 isolates) is represented on the horizontal axis, and the similarities between samples (measured as S_p coefficients) make up the vertical axis. Different samples are connected with horizontal lines at the similarity level they show to each others, and thus the higher up this line is, the more similar are the samples.

All data handling, including optical readings, calculations of correlation coefficients, diversity indices, and S_p values, as well as clustering and printing of dendrograms, were performed using the PhP software (BioSys inova, Stockholm).

RESULTS

Diversity and homogeneity of faecal coliform bacteria in piglets

The diversities of the faecal coliforms in six piglets and two sows are shown in Table 1. On the first sampling occasion the diversity of the bacterial flora of the sows was higher than that of most of the piglets (mean $D_i = 0·939$ and 0·540 respectively). The diversities within the piglets varied between samples and showed no special tendency to increase with age. A low diversity was often obtained in samples 3–5, which were collected during and soon after the diarrhoeal outbreak. Piglet P4, which had received antibiotics, showed a very low coliform diversity after that, and the diversity did not increase to normal levels until after the sixth sample. The two piglets that died during the outbreak carried a

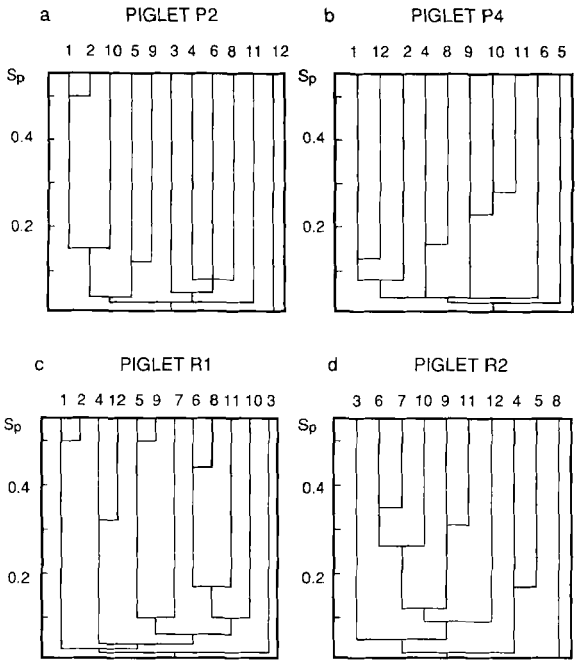

Figure 1. UPGMA clustering of S_p coefficients obtained from comparisons of intestinal coliform populations in four piglets (P2, P4, R1 and R2). The horizontal axes represent the number of the sample, and the vertical axes represent the similarities between different samples

coliform flora with a lower diversity than those that survived (mean D_j from samples 1–3 0·454 versus 0·744, respectively).

Persistence of the coliform flora in piglets

The coliform flora from different samples were compared within each piglet, and the corresponding S_p values were calculated. Figure 1 shows dendrograms derived from UPGMA clustering of the S_p matrixes obtained from four piglets which survived throughout the study.

Piglet P2 (Figure 1a). Samples 1 and 2 showed a high S_p value when compared to each other, indicating that the same coliform bacteria were present in those two samples. Sample 3 contained a coliform population totally different from that in the first two samples, and at all subsequent sampling occasions the S_p values were low, indicating that the coliform bacteria picked up at each sampling occasions were rapidly lost and not regained.

Piglet P4 (Figure 1b). The coliform flora changed between samples 1 and 2 in piglet P4. Unfortunately sample 3, at the onset of the diarrhoeal outbreak, was missing, but sample 4, collected after diarrhoea and antibiotic treatment, showed a very low S_p value, when compared to samples 1 and 2, indicating that the coliform bacteria from the first two sampling occasions had been lost. After that, an unstable coliform flora which changed between subsequent samples was observed up to sample 9, when the piglets were mixed, and a relatively stable coliform flora was obtained.

Piglet R1 (Figure 1c). This piglet also carried bacterial populations showing high S_p values to each other in samples 1 and 2. In sample 3, which was collected during the diarrhoeal episode, the coliform bacteria from the first two samples had been lost and replaced with new ones, which again were lost until the next sampling occasion. During the rest of the study, changes between subsequent samples were usually observed, but often bacterial strains from earlier sampling occasions were picked up again later (e.g. samples 4 and 12; 5 and 9; or 6 and 8).

Piglet R2 (Figure 1d). The first two samples were missing from piglet R2. Sample 3, collected during the diarrhoeal episode, contained a coliform flora different from all subsequent samples. Similar to the other piglets, an unstable coliform population with changes between samples was observed after sample 3 in this piglet.

Comparisons of the coliform floras between different piglets

During the first 2 wk of the study, the coliform flora usually differed between the piglets, even from the same litter, as well as between the sows and their piglets (data not shown). High S_p values were only obtained when sample 1 from piglets P3 and P4, and sample 2 from piglets P2 and P3 were compared (Table 2). In order to investigate whether coliform bacteria had caused the diarrhoeal outbreak at sampling occasion 3, the coliform populations of all piglets were compared for that particular sample (Figure 2). It was found that the piglets as well as their sow from litter R shared many identical coliform bacteria, whereas each piglet and the sow from litter P carried its coliform bacteria, which also were different from those of litter R. This is evidence that the organism that caused the diarrhoeal outbreak was not a member of the dominating coliform flora in the piglets.

Table 2. Similarities between the coliform floras from different piglets (P2, P3, P4, R1, and R2) and their sows (SP and SR). Only comparisons yielding S_p values higher than 0·20 are shown.

Sample no.	Compared piglets	S_p value
1	P3–P4	0·25
2	P2–P3	0·58
3	SR–R2	0·26
	R1–R2	0·33
4	SR–SP*	0·30
	SR–R1	0·43
5	—	
6	R2–P4*	0·20
7	—	
8	—	
9	R1–R2	0·29
	P2–P4	0·52
10	—	
11	R1–P2*	0·42
	R2–P4*	0·24
12	R1–P2*	0·43
	R2–P2*	0·48
	R2–P4*	0·49
	P2–P4	0·45

*Indicates comparisons between pigs originating from different litters.

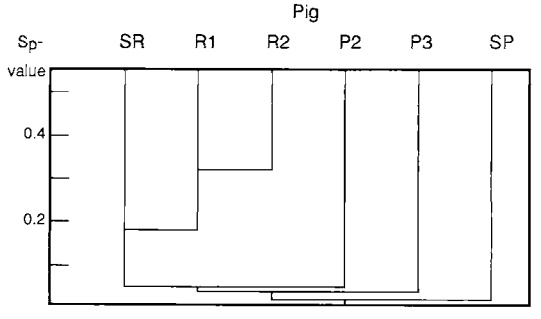

Figure 2. UPGMA clustering of S_p coefficients obtained from comparisons of coliform populations obtained from two sows (SP and SR) with two piglets each (R1, R2, P2 and P3). The sample was taken during an outbreak of diarrhoeal disease (sample no. 3). The vertical axis represents the similarities between different samples

At the earlier sampling occasions, before piglets from different sows were mixed, coliform populations showing high similarities to each other were sometimes found in piglets from the same sow, but very seldom in piglets from different sows (Table 2). After the piglets had been mixed with piglets from other litters (sample 10), bacterial floras from piglets of different sows sometimes showed high S_p values when compared (Table 2).

Dominant and resident coliform bacteria within piglets

When the biochemical fingerprints of isolates from all sows and piglets were compared to each other, altogether 215 different biochemical phenotypes (BPTs) were found. Most of these had colonised only one piglet and/or were found only on a single occasion, and could thus be regarded as transient strains. Thirteen major BPTs were found in several piglets, and in addition these BPTs were also often resident. Isolates belonging to these BPTs were species identified with the API 20E system. Twelve of these BPTs were *E. coli*, and one was *Citrobacter freundii*. The most common BPT was an *E. coli* strain accounting for 115 isolates. This BPT occasionally dominated the coliform intestinal flora of most of the piglets. However, it was not found in the piglets before the diarrhoeal outbreak, and was only found in 2 per cent of the isolates assayed from the outbreak.

DISCUSSION

The intestinal tract of humans and animals is normally sterile at birth, but it soon becomes colonised by microorganisms from the surrounding environment. Therefore we would expect a low diversity of bacterial populations sampled early in life from the piglets, which then increases with age. In the present study, the sows showed such an expected high diversity on the first sampling occasion. However, sample no. 1 collected from the piglets on the third day after birth already showed a high diversity in some piglets, e.g. piglet P4. There was no general tendency towards increase in the diversity in the subsequent samples, but merely fluctuation between low and high values. It is possible that the diarrhoeal outbreak complicated the pattern.

Piglets P3 and R4 died from diarrhoea during the outbreak. Both these piglets showed quite low diversities before that. This result supports previous findings, that a high diversity of the intestinal flora might suppress potential intestinal pathogens.[16] A decrease in diversity was also observed in sample 4 and 5 from both sows and their piglets. This may be due to effects of the antibiotic given during the diarrhoeal outbreak.

Diarrhoea in piglets is often caused by bacteria belonging to the Enterobacteriaceae group, mainly *E. coli*.[21] In a diarrhoeal outbreak in a pig herd caused by such bacteria we would expect to find identical bacterial strains in all infected pigs at high numbers, and thus high S_p coefficients between populations from different pigs. This was not the case in the present study. Although the piglets from litter R shared many identical coliform bacteria, each piglet from litter P carried its own unique coliform bacteria, different from those of litter R. Therefore, it is unlikely that the outbreak had been caused by coliform bacteria. This indicates that the PhP system may be a useful method for detecting virulent bacteria in an outbreak where the aetiological agents are unknown. If such an outbreak is caused by a specific coliform bacteria, diseased piglets will carry identical strains, which would be detected by PhP system.

An interesting finding was that the bacterial strains that had colonised the piglets immediately after birth did not persist for more than 2 weeks. It is believed that the first bacteria colonising newborn individuals easily become established, since there is no colonisation resistance from other bacteria.[17] In the present study, those three piglets which survived the diarrhoeal outbreak, and which were sampled from the third day of life, all contained different coliform populations in sample 4 compared to samples 1 and 2. Whether these changes of bacterial strains are normal events, or merely due to the diarrhoeal attack, is not known. We have previously shown that *E. coli* strains colonising newborn human infants immediately after birth persist for a longer time than those colonising them later in life.[9] We have also shown that there are certain coliform stains which are particularly efficient colonisers in newborn infants.[9,22] It is possible that the first strains colonising the piglets in the present study were not good enough colonisers, and therefore were later replaced with more efficient ones.

In conclusion, we showed that the PhP system for biochemical fingerprinting can be used to study diversities within and similarities between intestinal coliform populations. In the piglets studied here the diversity varied greatly between samples, but there was a decrease during and after a diarrhoeal outbreak, and piglets that died carried bacterial populations with lower diversity than those which survived the outbreak. We also showed that the coliform populations colonising the piglets soon after birth were lost later.

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