The Effect of Bambermycin, Carbadox, Chlortetracycline and Olaquindox on Antibiotic Resistance in Intestinal Coliforms: A New Animal Model

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SUMMARY

Groups of germ-free mice kept in isolators and associated with faecal microflora front piglets were continuously given either water or a solution of one of the following: chlortetracycline (20 µg/ml), carbadox (50 µg/ml), olaquindox (50 µg/ml), barnbermycin (flavomycin) (5 µg/ml) or mixtures of these drugs. The proportions of lactose-fermenting bacteria in their faeces which were resistant to chlortetracycline, carbadox or olaquindox were measured by a comparative plate-counting procedure. Compared to occurrence in control mice, the occurrence of antimicrobial drug-resistant bacteria was higher in mice receiving chlortetracycline ($P < 0.001$) and lower in mice receiving bambermycins ($P < 0.005$). In contrast, olaquindox and carbadox did not change the proportion of resistant coliforms in mice faeces. A control experiment was conducted with five groups of germ-free mice given the same flora and kept without drugs in separate isolators. No difference in the occurrence of resistant coliforms could be found between these groups. The germ-free mouse associated with faecal microflora from a conventional animal seems to be a suitable model for determining in vivo the effect of low doses of antimicrobial drugs on drug resistance in lactose-fermenting enteric flora.

Key-words: Growth promoter, Antibiotic resistance, Intestinal flora, Animal model; Axenic mice.
Abbreviations: BAM = bambermycin (flavomycine), DCA = deoxycholate agar, CAX = carbadox, OLX = olaquindox. CTC = chlortetracycline. SD = standard deviation.

INTRODUCTION

Sub-therapeutic levels of antimicrobial drugs are fed to livestock and poultry in order to increase the production of animal protein. However, the use of these agents has been restricted because of their undesirable effects on the gut flora: an increase in drug resistance among the intestinal *Enterobacteriaceae* is a frequent sequel to the use of certain antibiotic growth-promoters [10]. Numerous studies have been performed to deter-mine the effects of the continuous administration of diets containing low levels of antibiotics on the incidence of drug-resistant bacteria in the faeces of calves, pigs, chicken and dogs [2, 5, 6, 11, 15]. However, in these studies, the authors could not avoid (a) a high base-line level of resistant bacteria, (b) differences in flora between the animals at the start of the experiment [5] and (c) bacterial contaminations either from the diet [7], the water, the caretaker or between animals of control and experimental groups [11].

In order to overcome these experimental problems, we designed a new animal in order to study the effects of low doses of drugs on the drug resistance level in enteric micro-organisms: germ-free mice kept in isolators were associated with faecal flora front piglets and then were continuously given low doses of antimicrobial growth promoters used in piglet breeding. Total and drug-resistant coliform organisms were enumerated daily in the faeces of the mice. According to Ducluzeau et al. [3], who introduced focal flora of piglet into germ-free mice, the gross composition of the flora of the ex-germ-free mice was similar to that of donor piglets. Hazenberg et al. [8] obtained the same result in germ-free mice associated with human intestinal flora.

Chlortetracycline is well known to increase drug resistance among intestinal bacteria and, for this reason, its use in animal feeding has been prohibited in European countries [10, 17[but not in the USA. Since that time, new growth promoters have been synthesized and approved for use in swine feed: these include bambermycins, which decrease the percentage of focal Escherichia coli multiply-resistant to two of three antibiotics in pigs [2], and carbadox and olaquindox, quinoxaline dioxides which have been shown to have little or no effect on drug resistance level in the flora of pigs [6, 11]. The present studies were conducted to find a suitable small animal model in which to determine the effects of these antimicrobial drugs on the drug resistance characteristics of enteric flora.

MATERIALS AND METHODS

Experimental design.

A total of 84 adult female C3H germ-free mice provided by IFFA Credo (Lyon, France) were maintained in plastic isolators, fed ad libitum a solid milk replacer for piglets [1] which had been sterilized by irradiation (4 Mrad) and supplied with autoclaved drinking water. They were first divided into 5 unequal groups kept in separate isolators. The germ-free mice in each group, after having been deprived of water for 24h, were inoculated two consecutive days *per os* with a 1/100 dilution of piglet fresh faeces collected directly at the anus and given to the mice within 60 min after collection. The faeces dilution was made up just after collection in cold Schaedler broth (Bio-Mérieux, Marcy l'Étoile, France), thickened with 3 g/1 of Bacto agar (Difco), pre-reduced by boiling and kept under a paraffin layer. Faecal microflora numbers 0, 1, 2 and 3 (see table I) were taken from 6-day old piglets which had never received any drug. Flora no 4, came from a 30-day old piglet which was fed a commercial milk replacer containing 125 g of copper and 30 g of "Avoparcine" per ton (SAASO, Pibrac, France). Each group was then divided into experimental subgroups of four mice which were kept in separate mini-isolators [4]. The drinking water, free of drugs for the control groups, was supplemented with either carbadox (CAX) 50 µg/ml (Pfizer, New York, NY), olaquindox (OLX) 50 µg/ml (Bayer, Leverkusen, W. Germany), chlortetracycline (CTC) 20 µg/ml (Sigma, St-Louis, Mich.), bambermycin (BAM) 5 µg/ml (Flavomycine Hoechst, Frankfurt/Main, W. Germany), or mixtures of these drugs according to table I. The concentrations were equivalent to those given in food to livestock and poultry in general practice and the water intake of mice (4.1 ml/day) was similar to their food intake (4.7 g/day). Water was preferred to food for distributing the antibiotics to the mice because the drug solutions were easy to sterilize gently by filtration and because no data was available on antibiotic destruction by a 4-Mrad irradiation.

The drugs were introduced once a week in the isolators after having been sterilized by filtration on a 0.45µm pore filter. Because of its insolubility in water, CAX was dissolved in a small amount of 1N NaOH before filtration. The drugs were then diluted in the isolators with drinking water brought to pH 2.5 with HCl in order to avoid bacterial multiplication in the water and CTC degradation, and were thickened with 5 g/1 of Bacto Agar (Difco) to stabilize the suspension of CAX. The mice had been accustomed to the low pH of water for more than a month prior to the beginning of the experiment. Except for the first two days, their water consumption was normal. When the supplemented drinking water was assayed directly either by a disk-plate method using *Bacillus cereus* as the test organism (for CTC and BAM) or by spectro-photometry for CAX (pH brought to 12.5 with NaOH 1N (346 nm) and for OLX (373.3 nm), the following amounts of drugs were measured: CAX, 49.9±1.6 µg/ml; OLX, 52.2±3.4 µg/ml; CTC, 18.0±4.2 µg/ml; and BAM, 6.8±1.3 µg/ml (more than 15 assays were carried out for each drug). No detectable degradation of any of the drugs occurred after one week, since the drinking bottles of OLX had been darkened with ink to avoid photodestruction of OLX. Freshly passed faeces were collected directly at the anus and pooled for the four mice in one isolator. Samples were examined for resistant coliform organisms by a comparative plate counting technique within 30 min after the faeces had been collected. During the 6-week period of continuous drug administration, 2 to 5 samples were collected per week for the first three weeks, and one sample per week for the last three weeks. The number of sample collections is indicated in tables II to V.

Microbiological procedures.

A comparative plate-counting procedure was used to determine the incidence of CTC, CAX or OLX resistances in coliform organisms. Ten-fold dilutions of fresh faeces were made in sterile saline. Duplicate 0.1-m1 aliquots were plated on deoxycholate agar (DCA; Difco) and DCA + 25 µg of CTC per ml, DCA + 20 µg of CAX per ml or DCA + 10 µg of OLX per ml. These concentrations were chosen in accordance with usually accepted standards of resistance (CTC) or alter a preliminary study of the distribution pattern of minimal inhibitory concentrations (CAX and OLX) among piglet faecal *E. coli* strains. The total viable number of coliform organisms per gram of fresh faeces was determined from drug-free DCA plates and the number of resistant bacteria was determined from drug-supplemented media. Sixty isolated lactose-fermenting colonies, either from drug-free or from supplemented media, were sub-cultured and all of them gave a reaction typical of *E. coli*. Ten isolated colonies were picked from antibiotic-supplemented media and comparative counts of the liquid subcultures were made on Mueller-Hinton (Difco) and DCA, both supplemented with the same drug. In all cases, there were no significant differences between the numbers of colonies on the two media.

TABLE I. - Experimental diagram.

Flora from piglet No	Name of mice group	Drug given to mice	Mouse faeces enumerated on media
0	Control A	none	a, b (**)
	Control B	none	a, b
	Control C	none	a, b
	Control D	none	a, b
	Control E	none	a, b
1	Control 1	none	a, b
	CTC 1	CTC (*)	a, b
2	Control 2	none	a, c, d
	CAX 2	GAX	a, c
	OLX 2	OLX	a, d
3	Control 3	none	a, c, d
	CAX 3	CAX	a, c
	OLX 3	OLX	a, d
4	Control 4	none	a, b, c, d
	CTC 4	CTC	a, b
	CAX 4	CAX	a, c
	OLX 4	OLX	a, d
	BAM 4	BAM	a, b
	CTCCAX 4	CTC + CAX	a, b, c
	CTCOLX 4	CTC + OLX	a, b, d
	CTCBAM 4	CTC + BAM	a, b

Notes to table I: Each group was composed of four mice kept in a single cage in a separate isolator.
(*) Drinking water supplemented with: CTC = chlortetracycline (20 µg/ml), CAX = carbadox (50 µg/ml), OLX = olaquindox (50 µg/ml) or BAM = bambermycins (5 µg/ml).
(**) The pooled faeces of the four mice were enumerated on the following media: a = desoxycholate agar (DCA), b = DCA + CTC (20 µg/ml), c = DCA + CAX (20 µg/ml), d = DCA + OLX (10 µg/ml).

Transmission of drug resistance.

Transmissibility of drug resistance of *E. coli* was examined by mixed cultivation in brain heart infusion (Difco) for 8 h at 37°C. *E. coli* (K-12 Nal-resistant) was used as the recipient. The conjugation technique was tested with ten multi-resistant strains of *E. coli* from pig faeces. In these experimental conditions, ampicillin, chloramphenicol and streptomycin resistances were transmitted from seven strains to *E. coli* K12.

Twenty isolated colonies were picked from the CAX-supplemented plates used to count CAX-resistant coliforms in group 4 CTCCAX mouse faeces. Those donor strains were resistant to more than 50 µg/ml of CAX under aerobic conditions and more than 6.25 µg/ml under anaerobic conditions (Gas Pak, BioMerieux). Selection for the transconjugants was performed on Mueller-Hinton agar medium containing both nalidixic acid (25 µg/ml) and carbadox (20 µg/ml or 0.8 µg/ml whenincubated anaerobically). No transconjugant for CAX resistance could be found in these experimental conditions.

Statistical analysis.

Due to the wide range of percentages of resistant lactose-fermenting organisms in the mouse faeces, the results were expressed as decimal logarithms (log) of the number of total coliforms minus the log of the number of resistant coliforms. When skewness and kurtosis of these data were calculated, their distribution was found to be far from normal ($P<0.01$); for this reason, the non-parametric test of Wilcoxon [16] was conducted to determine which experimental groups were significantly different. Nevertheless, the standard deviations (SD) of the data were calculated and shown in the tables in order to give an indication of the dispersion of the experimental data.

A control experiment was conducted with 20 germ-free mice given the same flora and divided into five isolators without drug administration (table I, flora 0). The number of total and CTC-resistant coliforms were determined daily for 15 days in the pooled faeces of each group of mice, and isolators were compared in pair using Wilcoxon's test (table II). The determination of the number of CTC-resistant coliforms was chosen because, according to Linton et al. [12], it is related to the number of strains which are potential carriers of R plasmid.

RESULTS

Results reported in table II to V are means and SD of 10 to 20 logs of daily plate counts of pooled faeces from groups of four mice, each group kept in a separate isolator. The tables also give the means and SD of the differences, determined daily, between the log of the total number of coli-forms and the log of the number of resistant coliforms in the mice faeces.

Table II deals with CTC-resistant bacteria and shows the differences between five groups of mice 4noculated with the same microflora and kept without treatment in separate isolators. When the five isolators were compared two by two with Wilcoxon's test for the number of total coli-forms, 2 pairs out of 10 showed significant differences ($P < 0.001$) and, when compared for the number of resistant bacteria, 3 pairs out of 10 showed differences ($P < 0.005$). But when the isolators were compared by pair for the difference of log between total and resistant coliforms, no difference could be found at $P < 0.01$, and only one pair out of 10 was slightly different ($0.05 > P > 0.02$), as can be seen in table II.

TABLE II. Differences of flora between five groups of germ-free mice associated with the same microflora, each kept in a separate isolator without drug administration.

Isolators	Total nb of coliforms per g of faeces		Difference of log between total and CTC-resistant coliforms		Wilcoxon's test: differences between isolators taken by pair				
	log	SD	Log	SD	B	C	D	E	
A	7.35	0.39 (1)	2.13	0.58 (2)	= = = (3)	= = =	= =	= = =	.
B	7.75	0.50	2.18	0.65		= = =	= =	= = =	.
C	7.38	0.40	2,26	0.68			=	= = =	.
D	8.05	0.20	1.83	0.36				= = =	.
E	7.73	0.50	2.04	0.55					

Notes to table II

(1) Mean and standard deviation (SD) of 13 log of daily plate counts on DCA over a 15-day period.

(2) Mean and SD of 13 differences of log between total and CTC-resistant coliforms, enumerated on DCA + CTC (25 µg/ml).

(3) Wilcoxon's test, based on 13 daily differences between two isolators, for the difference of log between total and resistant coliforms. === : $P > 0.1$, == : $P > 0.05$: = : $P > 0.02$.

Table III shows the effect of 50 µg of OLX per ml of drinking water on the occurrence of OLX-resistant bacteria in the faeces of germ-free mice associated with three different piglet microfloras. The proportions of OLX resistant bacteria were low in the three floras, ranging from 0.026 to 0.42%. No significant difference ($P > 0.10$) could be found in the differences of log between total and resistant coliforms for any of the three flora between control and OLX-treated groups. The addition of 20 µg of CTC to the OLX in the drinking water (isolator CTCOLX 4) produced had no further effect on the proportion of OLX-resistant coliforms ($P > 0.10$), as can be seen in table III.

TABLE III. - Influence of olaquindox on OLX-resistant coliforms in the flora of germ-free mice associated with piglet microflora.

Isolalor and flora	Nb of counts	Total Nb of coliforms /g faeces		Difference of log between total and OLX-resistant coliforms		Wilcoxon's test: Difference between control and treated groups
		log	SD	log	SD	
Control 2 (1)	19	8.82	0.37 (2)	2.55	0.86 (3)	
OLX 2	19	8.70	0.31	2.38	0.71	= = = (4)
Control3	19	8.84	0.36	2.75	0.79	
OLX 3	19	8.63	0.27	2.61	0.57	= = =
Control4	11	7.93	0.70	3.58	0.96	
OLX 4	11	7.74	1.05	2.65	1.02	= = =
CTCOLX 4	71	7.65	0.89	3.26	0.96	= = =

(1) Drug treatment and microflora given: see table I.
(1) Mean and SD of 19 (floras 2 and 3) or 11 (flora 4) logs of daily plate counts on DCA.
(3) Mean and SD of 19 (or 11) differences of log between enumerated on DCA + OLX (10 µg/ml).
(4) See table II.

Table IV shows the influence of 50 µg of CAX per ml of drinking water on the occurrence of CAX-resistant coliforms in the faeces of germ-free mice associated with three different floras. The proportions of CAX resistant coliforms were low, ranging from 0.012 to 0.19%. No significant difference could be found in the resistance under CAX administration for floras 2 and 4 ($P > 0.10$), and a slightly significant increase in resistance was obtained in flora 3 ($P = 0.02$). In contrast, the proportion of CAX-resistant coliforms was high in the CTCCAX 4 group (13.3 %) and the difference between the control group and that treated with a mixture of CTC and CAX was significant ($P < 0.001$).

TABLE IV. - Influence of CAX on the CAX-resistant coliforms in the flora of germ-free mice associated with piglet microflora.

Isolator and flora	Nb of counts	Total nb of coliforms /g faeces		Difference in log between total and CAX-resistant coliforms		Wilcoxon's test: difference between control and treated group
		Log	SD	Log	SD	
Control 2 (1)	19	8.82	0.37 (2)	3.39	0.99 (3)	
CAX 2	19	8.87	0.30	3.34	0.75	= = =(4)
Control3	19	8.84	0.36	4.11	1.11	
CAX 3	19	8.93	0.29	3.75	1.01	=
Control4	12	7.73	0.83	2.64	0.53	
CAX 4	12	8.01	1.13	2.77	1.35	= = =
CTCCAX 4	12	8.46	0.88	0.88	0.67	* * *

(1) Drug treatment and given microflora : see table I.
(2) Mean and SD of 19 (floras 2 and 3) or 12 (flora 4) log of daily plate counts on DCA.
(3) Mean and SD of 19 (or 12) daily differences of log between total and CAX-resistant coli-forms enumerated on DCA + CAX (20 µg/ml).
(4) see table II; * * * : $P < 0.001$.

Table V shows the influence of CTC, BAM and mixtures of CTC with BAM, CAX, or OLX on the proportion of CTC-resistant coliforms in the faeces of germ-free mice associated with two different piglet microfloras. The proportions of CTC-resistant coliforms were quite different in the two control groups (0.52% for n° 1 and 33.9% for n° 4), but the addition of 20 µg CTC per ml of water significantly increased the proportion of resistant coliforms ($P<0.001$) in both flora (15% CTC-resistant for CTC l, 100% for CTC 4). On

the contrary, BAM (5 µg per ml of drinking water) decreased, from 33.9% to 5.1%, the proportion of resistant coliforms (P <0.005) in faeces, as can be seen in table V. The proportion of drug-resistant coliforms in the faeces of mice given both CTC (20 µg/ml) and BAM (5 µg/ml) was not different from the proportion found in controls (P >0.10), but was higher than in BAM-treated mice (P <0.005) and slightly lower than in CTC-treated mice (P=0.03). The association of CTC with CAX or OLX in the water had not significant effect on the proportion of CTC-resistant coliforms either when compared with control 4 (P >0.05 for CAX or P >0.10 for OLX) or when compared with CTC-treated mice CTC 4 (P >0.05 for CAX or P >0.10 for OLX).

TABLE V. - Influence of CTC, BAM or mixtures of CTC and others drugs on CTC-resistant coliforms in the flora of germ-free mice associated with piglet microflora.

Isolator and flora	Nb of counts	Total nb of coliforms /g faeces		Difference in log between total and CTC-resistant coliforms		Wilcoxon's test: difference between control and treated group
		Log	SD	Log	SD	
Control 1 (1)	20	9.25	0.62 (2)	2.28	0.40 (3)	
CTC 1	19	9.61	0.17	0.80	0.39	* * * (4)
Control 4	13	7.87	0. 69	0.47	0.27	
CTC 4	13	7.88	0.97	-0.09	0.43	* * *
BAM 4	13	7.03	0.37	2.29	1.55	* *
CTCBAM 4	11	7.74	0.40	0.33	0.20	= = =
CTCCAX 4	12	8.46	0.88	0.25	0.29	= =
CTCOLX 4	10	7.65	0.89	0.26	0.31	= = =

(1) Drug treatment and given flora: see table I.
(2) Mean and SD of 20 (flora 1) or 10 to 73 (flora 4) log of daily plate counts on DCA.
(3) Mean and SD of 20 to 10 differences in log between total and CTC-resistant coliform enumerated on DCA + CTC (25 µg/ml).
(4) See. table II, * * *: P < 0.001, **: P < 0.005.

DISCUSSION

In these studies, the occurrence of antimicrobial drug-resistant lactose-fermenting bacteria was higher in mice receiving CTC and lower in mice receiving BAM than in the control groups. In contrast, OLX and C AX did not change the proportion of resistant coliforms in mice faeces. These results are similar to those of numerous authors, who studied the effect of low doses of tetracyclines [11, 14, 15], BAM [2], CAX [6] and quindoxin, which is also a quinoxaline dioxide [11], in the faecal microflora of calves, pigs, chickens and dogs. In mice receiving mixtures of CTC and one other drug (i.e., BAM, CAX or OLX), the occurrence of CTC-resistant coliforms did not differ front that of the control group, and this may be related to the elimination of R factors in *E. coli* demonstrated in vivo for BAM by Dealy and Moeller [2] and in vitro for CAX by Gedek [6]. On the other hand, the mixture of CAX and CTC increased the proportion of CAX-resistant coli-forms, and this may be related to the R plasmid carrying resistance to CAX and other drugs demonstrated in pigs by Ohmae et al. [13], although we could not evidence such a transmissible resistance in *E. coli* strains from mice faeces.

Five prerequisites for a model in which to determine the effects of an antimicrobial drug on drug resistance in the aerobic enteric flora are (a) a low base-line of resistance [11], (b) identity in the level of resistance between the groups of animals at the start of the experiment [5], (c) absence of contamination during the trial, especially with resistant bacteria from diet [7] or water, the caretaker, or between the experimental groups of animals [11], (d) low variability over time in the percentage of resistant bacteria in the control flora and (e) an animal system in which the observed responses from use of a spectrum of antimicrobial drugs are in accordance with already published data.

In the present study, these five criteria were met.

a) The level of resistance in the lactose-fermenting enteric flora n° 0 to 3 was low and decreased with time in the control animals, as can be seen in kinetics presented in a previous paper [1]. Microflora n° 4 was obtained from a piglet given an antibiotic-supplemented diet and showed a high proportion of resistant coliforms at the start of the experiment (table V), but this was an advantage in testing the action of BAM.

b) The identity of the flora between different groups of mice given the same complex flora but kept in separate isolators was evidenced in the experiment reported in table II.

c) The use of isolators guaranteed freedom of contamination from other groups of mice or from men, and the acidified water prevented the multiplication of *Enterobacteriaceae* in the drinking water.
d) The variability over time of the data analysed here was lower ($P < 0.05$ by F test) in mice (SD=0.56, flora 0, from 13 daily counts over a 15-day period) than in the donor animals (SD=1.30 during the same period).
e) The observed responses from use of CTC, BAM and CAX were similar to those obtained by authors working with pigs or calves.
The germ-free mouse may be associated with complex or simplified flora from any monogastric animal or human. The similarity of the dominant bacterial species of the faecal flora between the donor animal and the inoculated mice has already been checked [1, 3, 8]. Due to species differences other than the flora, i.e. drug absorption or the enterohepatic cycle, direct extrapolation to animals or humans should not be considered. However, if one keeps in mind that it is only an animal model (like rodents in toxicology), the germ-free mouse associated with complex floras seems to be a valuable tool to study in vivo the effects of drugs on bacteria: Hazenberg et al [9] used such a model to study the effect of therapeutic doses of sulphasalazine on human flora. The results presented here are in accordance with those of other authors. The proposed model therefore seems to be a suitable tool to test the action of low doses of amtibiotics, since it is free of in any interfering factors. However, in a real » life, other factors may operate, and the model can be used complementary to monitoring in the normal host.

RÉSUMÉ

Effet de la flavomygine, du carbadox, de la chlortétracycline et de l'olaquindox sur l'antibio-résistance des coliformes fécaux : un nouveau modèle animal

Des souris axéniques inoculées avec la microflore fécale de porcelets et maintenues en isolateur, ont reçu en apport continu dans leur eau de boisson, seuls ou en association, les facteurs de croissance suivants : carbadox (50 µg/ml), chlortétracycline (20 µg/ml), flavomycine (5 µg/ml) et olaquindox (50 µg/ml). Les proportions de coliformes résistants à la chlortétracycline, au carbadox et à l'olaquindox dans les selles de ces souris, ont été déterminées pendant plusieurs semaines par des numérations sur des milieux sélectifs. Par rapport à un groupe de souris témoin, la chlortétracycline a augmenté la proportion de bactéries résistantes ($P < 0,001$) alors que la flavomycine a fait baisser cette proportion ($P < 0,005$). Ni le carbadox ni l'olaquindox n'ont eu d'effet significatif. Par ailleurs, dans une expérience de contrôle, aucune différence dans la proportion des coliformes fécaux résistants n'a été trouvée entre cinq groupes de souris qui avaient reçu la même flore et buvaient de l'eau sans additif. La souris axénique associée à une microflore complexe venant d'un animal d'élevage, semble un modèle convenable pour l'étude in vivo de l'effet de faibles doses de facteurs de croissance sur la résistance aux antibiotiques des coliformes fécaux.

MOTS-CLÉS : Additif alimentaire, Antibiorésistance, Microflore intestinale, Modèle animal ; Souris axénique.

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