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Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*

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***Objectives*: To determine the level of antimicrobial resistance and the occurrence of class 1, 2 and 3 integrons in faecal *Escherichia coli* from several animal populations variously exposed to human contact.**

***Methods*: A collection of 341 faecal *E. coli* isolates was constituted from several animal populations subject to various degrees of exposure to humans: 18 animals never exposed to humans (living in the Antarctic or Gabon), 71 wild animals living in a low human density area (mountainous region of the Pyrenees, France), 61 wild animals living in a higher human density area (Fontainebleau forest near Paris, France), and 128 extensively reared farm animals and 42 pet dogs, both living in the Pyrenees. Resistance to antimicrobial agents was determined by the method of disc diffusion and quantified using the resistance score of BE Murray, JJ Mathewson, HL DuPont, CD Ericsson and RR Reves (Antimicrobial Agents and Chemotherapy 1990; 34: 515–18). Integrons were characterized by triplex real-time PCR and sequencing. The absence of epidemiologic clones was confirmed by PCR-based methods.**

***Results*: A gradient of resistance ranging from absence to high prevalence (resistance score of 18.7%) and a gradual increase in the prevalence of class 1 integrons (from 0% to 16%), both correlated with the increase in exposure to humans, were observed. In wild animals with little contact with humans, resistance, when present, was not mediated by integrons.**

***Conclusions*: Our findings firmly establish that the current prevalence of antimicrobial resistance found in animal faecal bacteria, as well as the prevalence of integrons, is clearly anthropogenic. The presence of integrons may constitute an adaptive process to environments whose antimicrobial pressure exceeds a certain threshold.**

Keywords: selective pressure, environment, wild, domestic, pets

Introduction

Animal and human commensal microbiota, especially intestinal microbiota, are subjected to numerous antimicrobial pressures due to farming practices and veterinary and human medicines. They have been suggested to play a major role in disseminating bacterial resistance. Indeed, an enormous number of bacterial species are present at high density in an environment allowing gene exchange between bacteria, and these bacteria can disseminate among ecosystems via the contact between humans and animals, the food chain and the recycling of animal waste as fertilizer. A few studies have concomitantly documented the prevalence of multiresistant bacteria and integrons, highly efficient molecular tools used by the bacteria for antimicrobial resistance acquisition and expression, in human commensal microbiota. They showed that, although at a lower rate than in clinical isolates, both are clearly present, with an integron prevalence of about 15%.[1,2] Several authors have documented a high prevalence of integrons in intensive reared farm animals, ranging from 23% to 44%,[1,3] a level comparable to the prevalence found in human clinical isolates.[4] Some studies have been devoted to comparing the level of antimicrobial resistance in bacteria

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isolated from the faeces of wild animals with the level in animals in contact to humans.[5,6] However, no definitive conclusions were reached since the prevalence of antimicrobial resistance was variable according to the studies. The only data available on integron prevalence in wild animals were described in strains (n = 48) isolated from free-living geese, with a range of 0% (geese not near animal food facilities) to 36% (geese interacting with swine waste lagoons).[7]

In this work, we have surveyed the prevalence of acquired antimicrobial resistance and characterized the integrons in faecal *Escherichia coli* isolated from several animal populations subject to various degrees of exposure to humans.

Materials and methods

E. coli were isolated between 2001 and 2003 from fresh faeces collected from (i) 150 wild birds and mammals, (ii) 128 extensively reared farm animals and (iii) 42 pet dogs. The 150 wild animals can be classified into three distinct populations, according to their human contact, as follows: a population of 18 living in areas with human densities ≤1 inhabitant per km^2, including the Antarctic (n = 4) and pristine forest in central Gabon (n = 14); a population of 71 in the middle mountain region of the central Pyrenees, France, with <50 inhabitants per km^2; and a population of 61 in the Fontainebleau forest near Paris, France, with 200 inhabitants per km^2. Both the 128 farm animals and 42 pet dogs were living in the central Pyrenees. Some of the farm animals were epidemiologically related as these animals lived in five different farms and as in each farm 1–6 animals by species were studied. A full description of the animal species studied is given in Table S1 [available as Supplementary data at *JAC* online (http://jac.oxfordjournals.org/)]. The isolates were identified as *E. coli* using API 20E strips (API, La Balme-les-Grottes, France). One *E. coli* colony per sample was studied, except in the Antarctic and Gabon populations, in which one to three distinct isolates per animal were analysed (39 isolates for 18 animals). In these populations, isolates from a single animal were considered as distinct if they belonged to different *E. coli* phylogenetic group/subgroups, determined using triplex PCR (see below).

Strains were assigned to the seven *E. coli* phylogenetic group and subgroups using triplex PCR based on the presence or absence of three DNA fragments (*chuA*, *yjaA* and TSPE4.C2) as described by Escobar-Paramo *et al.*[8] In addition, the presence of four virulence determinants (*pap, sfa, hly* and *aer*) was tested by PCR, as described previously.[9]

Antimicrobial susceptibilities were determined by the method of disc diffusion, according to the guidelines of the French Society of Microbiology antibiogram committee (CASFM, www.sfm.asso.fr). The seven resistance markers tested were representative of the spectrum of mechanisms leading to antimicrobial resistance and included amoxicillin, sulfamethoxazole, chloramphenicol, kanamycin, streptomycin, tetracycline (for plasmidic resistance) and nalidixic acid (for chromosomal resistance). When a strain carried an integron, the marker trimethoprim was also tested.

Class 1, 2 and 3 resistance integrons were detected and characterized as described previously by Skurnik *et al.*[2]

Significant differences between groups were determined by the χ^2 test using the R statistical system software (www.r-project.org). $P < 0.05$ was considered significant.

Results and discussion

Isolates from animals never exposed to humans (living in the Antarctic or Gabon) were totally free of resistance. Of the isolates from wild animals living in the low human density area (mountainous region of the central Pyrenees) 17% were resistant to at least one antimicrobial, versus 49% of isolates from wild animals living in a higher human density area (Fontainebleau forest near Paris) (χ^2: $P < 0.001$). No difference in the prevalence of resistance to at least one antimicrobial was observed in the *E. coli* isolated from the wild animals in the Fontainebleau area, the farm animals or the pets. However, when resistance scores (calculated as the ratio of the total number of observed resistance to the total number of possible resistance × 100)[10] reflecting multiresistance to antimicrobials were considered, the isolates from farm animals were more resistant than those from wild animals (χ^2: $P < 0.001$), but less resistant than those from pets (χ^2: $P < 0.001$). As an element of comparison, the isolates from pets exhibited a similar level of resistance to that of isolates from 49 healthy French subjects working in the bank or insurance sector who had taken no antimicrobials during the month before sampling (Figure 1).[2] Integrons were only found in *E. coli* isolated from the animals living in close contact with humans: farm animals (7%) and pets (16%). All integrons were of class 1 only. For comparison, the prevalence of integrons in the *E. coli* faecal flora of the French subjects was 16% (Figure 1).[2] Characterization of the integrons from the animal strains showed strikingly little diversity in their gene cassette content (Table 1). This contrasted with the great heterogeneity of the integrons previously found in clinical isolates,[4] but was in agreement with what had been observed in human commensal isolates, in which all the gene cassettes were derivatives of *aadA* or *dfrA*.[2] As regards the integrons identified in the strains from farm animals, the low diversity of their cassettes was even more pronounced than what was reported for human commensal isolates,[2] because all the integrons except one carried the same gene

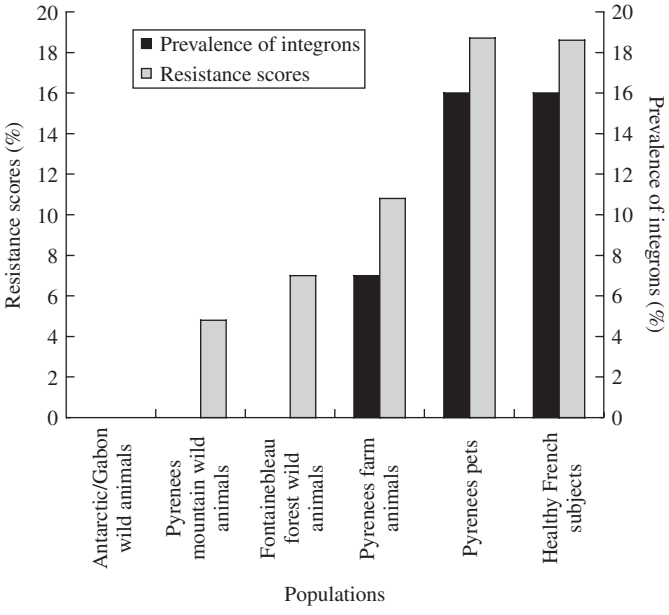

Figure 1. Resistance scores and prevalence of integrons in faecal *E. coli* isolates from wild animals living in areas with different human densities, i.e. the Antarctic/Gabon (n = 39), central Pyrenees, France (n = 71), and the Fontainebleau forest near Paris, France (n = 61), and from extensively reared animals (n = 128) and pet dogs (n = 42), both from the central Pyrenees, compared with commensal *E. coli* isolated from healthy French subjects (n = 49) and studied by the same approach.[2]

Table 1. Strain patterns and integron characteristics of the resistant animal faecal *E. coli* isolates

Strain pattern[a]				Integron characteristics				
strain pattern number	phylogenetic group and subgroups	virulence gene	antibiotic resistance phenotype[b]	n[c]	gene cassette	fragment size (bp)	host	source
I (n = 2)[d]	A_0	no	AMX	0				
II (n = 4)	A_0	no	TET	0				
III (n = 1)	A_0	no	AMX/SUL/TET	0				
IV (n = 2)	A_0	no	SUL/STR/TET	2	none/ *dfrA1-aadA1*	100/1200	*Oryctolagus cuniculus* (rabbit)/*Gallus gallus* (chicken)	farm
V (n = 2)	A_1	no	KAN	0				
VI (n = 14)	A_1	no	TET	0				
VII (n = 1)	A_1	no	AMX/TET	1	*sat1-aadA1*	1300	*Sus scrofa domestica* (pig)	farm
VIII (n = 1)	A_1	no	SUL/STR/TET	0				
IX (n = 1)	A_1	no	AMX/STR/TET	0				
X (n = 1)	A_1	no	SUL/STR/TET	0				
XI (n = 2)	A_1	*aer*	AMX/SUL/ STR//TET	2	*dfrA1-aadA1/ dfrA1-aadA1*	1200/1200	*Canis familaris* (dog)/*Canis familaris* (dog)	pets
XII (n = 1)	B1	no	AMX	0				
XIII (n = 15)	B1	no	TET	0				
XIV (n = 1)	B1	no	AMX/TET	0				
XV (n = 3)	B1	no	AMX/SUL/TET	1	*dfrA15*	500	*Canis familaris* (dog)	pet
XVI (n = 1)	B1	no	AMX/STR/TET	0				
XVII (n = 4)	B1	no	SUL/STR/TET	2	*aadA1/ dfrA1-aadA1*	800/1200	*Gallus gallus* (chicken)/*Equus caballus* (horse)	farm
XVIII (n = 1)	B1	no	AMX/SUL/ STR/TET	1	*dfrA1-aadA1*	1200	*Sus scrofa domestica* (pig)	farm
XIX (n = 1)	B1	*hly*	TET	0				
XX (n = 1)	B1	*hly, aer*	TET	0				
XXI (n = 1)	B1	*pap*	AMX/SUL/ CHL/TET/NAL	0				
XXII (n = 1)	B1	*pap*	AMX/SUL/CHL/ STR/TET/NAL	0				
XXIII (n = 1)	B1	*pap, aer*	AMX/SUL/CHL/ TET/NAL	0				
XXIV (n = 1)	B1	*pap, aer*	AMX/SUL/CHL/ STR/TET/NAL	1	*aadA1*	800	*Canis familaris* (dog)	pet
XXV (n = 5)	$B2_2$	no	TET	0				
XXVI (n = 1)	$B2_2$	no	AMX/SUL/KAN/ STR/TET	0				
XXVII (n = 1)	$B2_2$	no	AMX/SUL/CHL/ STR/TET	0				
XXVIII (n = 1)	$B2_2$	*sfa, hly*	SUL/STR	0				
XXIX (n = 1)	$B2_2$	*pap, sfa, aer*	TET	0				
XXX (n = 9)	$B2_3$	no	TET	0				
XXXI (n = 1)	$B2_3$	*aer*	AMX/TET	0				
XXXII (n = 1)	$B2_3$	*aer*	AMX/SUL/TET	1	*dfrA15*	500	*Canis familaris* (dog)	pet
XXXIII (n = 1)	$B2_3$	*pap, hly*	TET	0				
XXXIV (n = 1)	$B2_3$	*pap, sfa*	TET	0				

Table 1. (*continued*)

Strain pattern[a]				Integron characteristics				
strain pattern number	phylogenetic group and subgroups	virulence gene	antibiotic resistance phenotype[b]	*n*[c]	gene cassette	fragment size (bp)	host	source
XXXV (*n* = 1)	$B2_3$	*pap, sfa, aer, hly*	AMX/NAL	0				
XXXVI (*n* = 23)	D_1	no	TET	0				
XXXVII (*n* = 1)	D_1	no	SUL	1	*sat1-aadA1*	1300	*Equus caballus* (horse)	farm
XXXVIII (*n* = 3)	D_1	no	AMX/TET	0				
XXXIX (*n* = 1)	D1	no	AMX/SUL	0				
XL (*n* = 1)	D_1	no	SUL/STR	1	*aadA1*	800	*Bos taurus* (cow)	farm
XLI (*n* = 1)	D_1	no	STR/TET	0				
XLII (*n* = 1)	D_1	no	AMX/SUL/STR/TET	1	*dfrA7*	500	*Canis familaris* (dog)	pet
XLIII (*n* = 1)	D_1	no	AMX/SUL/CHL/TET/NAL	1	*dfrA1-aadA1*	1200	*Canis familaris* (dog)	pet
XLIV (*n* = 1)	D_1	*hly*	AMX/SUL/CHL	0				
XLV (*n* = 2)	D_2	no	TET	0				
XLVI (*n* = 1)	D_2	no	STR/TET	0				
XLVII (*n* = 1)	D_2	no	SUL/STR/TET	1	*aadA1*	800	*Sus scrofa domestica* (pig)	farm
XLVIII (*n* = 1)	D_2	*aer*	AMX/SUL/CHL/TET/NAL	0				
XLIX (*n* = 1)	D_2	*aer*	AMX/SUL/CHL/STR/TET/NAL	0				

[a]The strain pattern is defined as the association of the *E. coli* group/subgroup, the virulence gene content and the antibiotic resistance phenotype.
[b]AMX, amoxicillin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol; TET, tetracycline; SUL, sulfamethoxazole; NAL, nalidixic acid.
[c]*n*, Number of strains carrying an integron in each strain pattern.
[d]*n*, Number of isolates in each strain pattern.

cassette: *aadA1*, either alone or with *dfrA1* or *sat1* (Table 1). The only integron found without *aadA1* in an *E. coli* strain isolated from a farm animal was in fact an 'empty' integron, with 5′ and 3′ conserved segments, but without gene cassettes. Such empty integrons have been found in coliform bacteria isolated from an aquatic environment and seem to be indicators of the absence of sustained antimicrobial pressures.[11,12] As regards the integrons found in our pet isolates, the gene cassette distribution was very similar to the one we previously found in human commensal isolates.[2] When all the resistance and integron data were taken into account, no difference was observed between the commensal pet and human isolates, suggesting that they were subject to the same selective pressure.

To determine whether the presence of resistant isolates in different ecosystems was caused by the spread of certain clones or of resistance genes, we analysed the membership of the seven *E. coli* phylogenetic group/subgroups as well as the virulence gene content profile of the isolates (Table 1). We found that the strain patterns were extremely heterogeneous, suggesting that, as found by others,[1] the presence of resistant strains and of integrons in the different ecosystems of our study was not due to the spread of specific clones. Of note, among resistant isolates, only one strain belonging to the B2 phylogenetic group carries an integron (Table 1).

In all, we observed a gradient of resistance ranging from absence to high prevalence (resistance score > 18%); this gradient correlated with the level of exposure to humans and/or human activities. We also found a link between the presence of integrons and exposure to humans. In France, the consumption of antimicrobials is 36.5 Defined Daily Dose/1000 inhabitants/day, and 1382 tonnes of antibiotics, half of them consisting of tetracycline, are consumed each year in veterinary medicine. Therefore, exposure to humans and farming activities can probably be associated with increased antimicrobial selective pressure. The fact that in the Pyrenees the difference between the resistance scores for wild and farm animals (5% versus 11) (Figure 1) almost disappeared when resistance to tetracycline was removed from the data (4% versus 5) strengthens this hypothesis. We noted with interest that the traditional link between integron prevalence and antibiotic resistance[4] was present in the *E. coli* isolated from pets and farm animals, but not in resistant strains from the wild animals, which had less contact with humans and/or human activities, as reported for free-living geese.[7] Thus, even though strains were still resistant, integron prevalence decreased and then disappeared as contact with humans decreased. This suggests that the presence of integrons may constitute an adaptive process to environments whose antimicrobial pressure exceeds a certain threshold.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* online (http://jac.oxfordjournals.org/).

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