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ORIGINAL ARTICLE

Differentiation of faecal *Escherichia coli* from humans and animals by multiple antibiotic resistance analysis

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Abstract

Aims: Multiple antibiotic resistance (MAR) was performed on 128 *Escherichia coli* isolates, recovered from faecal samples of humans and animals (cattle, goat, sheep) to determine and compare their antibiotic resistance patterns and to evaluate them statistically in order to specify the source of the faecal material.
Methods and Results: Disk diffusion method was applied with a selection of antibiotics. Statistical approach was performed with hierarchical cluster analysis (CA), discriminant analysis (DA) and principal component analysis. Comparing human and animal isolates there was significant difference in levels of resistance to all antibiotics tested ($P < 0{\cdot}05$) with 46 and 24 distinct resistance patterns for human and animal isolates respectively. CA and DA separated human and animal isolates with a high average rate of correct classification (99·2%), when all animal isolates were pooled together.
Conclusions: MAR analysis compared with appropriate statistical evaluation may provide a useful tool for differentiating the human or animal origin of *E. coli* isolates derived from environmental samples. Subsequently, determination of the source of faecal pollution becomes possible.
Significance and Impact of the Study: Determining the source of faecal pollution enables the prediction of possible risk for public health and the application of appropriate management plans for prevention of further contamination.

Introduction

Faecal pollution can be a problem for estuaries that are associated with wildlife and human populations as it degrades water quality and restricts its use (Parveen *et al.* 2001). However, without knowing the precise source of faecal input, the human health risk cannot be predicted accurately (Parveen *et al.* 1999). Faecal material from humans indicates the possible presence of human enteric pathogens such as *Salmonella* spp. or hepatitis A virus (Guan *et al.* 2002), while less risk is posed by nonhuman faecal material as the aforementioned pathogens are mainly associated with human diseases. Thus it would be desirable to determine the source of the faecal material, especially if monitoring and management plans for prevention of further contamination are to be developed (Harwood *et al.* 2000).

Several attempts have been made to determine the sources of faecal material, including the ratio of faecal coliforms to faecal streptococci (Wiggins *et al.* 1999), detection of specific bacteriophages (Hagedorn *et al.* 1999), study of phenotypic (Stefani 2000), genetic structure and diversity of populations of faecal coliforms (Souza *et al.* 1999; Galland *et al.* 2001; Georgnaras *et al.* 2001) and patterns of antibiotic resistance in certain populations of micro-organisms (Gonzalo *et al.* 1989; Mathew *et al.* 1999; Chee-Sanford *et al.* 2001).

No single method has yet emerged as the best one and there is a clear lack of comparative studies to determine the relative strength and weakness of each method

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(Graves *et al.* 2002). Among the most traditional methods is multiple antibiotic resistance (MAR), which has already been used to differentiate sources of faecal pollution (Parveen *et al.* 1999). Despite its limitations of unstable resistance (i.e. plasmids), geographical variation and limited specificity it has been reported in many studies that this approach is certainly a simple and cost-effective method for most laboratories (Parveen *et al.* 2001).

Most investigations concerning antibiotic resistance, focus on bacteria of faecal origin because they are commonly used as indicators of faecal pollution in water and food (Harwood *et al.* 2000) and may be associated with infectious diseases (Goñi-Urriza *et al.* 2000). Specifically, *Escherichia coli* is routinely employed as an indicator of the potential presence of human enteric pathogens (Parveen *et al.* 1999), but it is not limited to humans as it inhabits the gastrointestinal tract of many warm-blooded animals as well, being implicated in human and animal infectious diseases. Therefore, determination of certain characteristics of that indicator may provide data concerning the sources of pollution.

The objectives of this study were: (i) to determine and compare antibiotic resistance patterns in *E. coli* isolates recovered from human and animal faecal samples, with antibiotics commonly used in veterinary practice and human treatment and (ii) to evaluate statistically the distinct patterns of antibiotic resistance for the possible specification of the source of the faecal material. For this purpose cluster analysis (CA) and discriminant analysis (DA) were performed, as they have already been proposed for the possible differentiation between animal and human sources of *E. coli* (Hagedorn *et al.* 1999; Harwood *et al.* 2000).

Materials and methods

Bacterial strains

In total, 128 *E. coli* isolates were collected directly from fresh human and animal faeces according to standard procedures (Parveen *et al.* 1997). Human faecal samples were obtained from healthy volunteers and hospitalized patients with gastroenteritis (children and adults). Animal faecal samples were obtained from the local veterinary department of Ministry of Agriculture and Food. Animal faeces came from numerous farms breeding cattle, goats and sheep (Table 1). All faecal samples (human and animal) were derived from the geographical area of Achaia District, located south-west of Greece and were collected from September 2000 to March 2001.

Faecal samples were diluted in 10-fold serial increments and 0·1 ml of each dilution was plated on MacConkey agar (Becton Dickinson, Sparks, MD, USA) and incubated at 37°C for 18–20 h. Typical *E. coli* colonies were screened on Tryptone Bile X-Glucoronide Medium-TBX (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 44°C for 18–20 h. *Escherichia coli* isolates were confirmed, using standardized identification system API 20E (bioMérieux, Marcy-l'Étoile, France).

Table 1 *Escherichia coli* isolates obtained from animal and human faeces

Origin of *E. coli* strain	No. of isolates
Cattle	14
Sheep	24
Goat	22
Human	
Adult patients	39
Paediatric patients	21
Healthy	8
Total	128

Antibiotics

Thirteen antibiotics were tested. Their corresponding disk quantities were as follows: amikacin (30 μg), amoxicillin (25 μg), ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), neomycin (30 UI), nitrofurantoin (300 μg), norfloxacin (10 μg), oxytetracycline (30 μg), sulfathiazole (0·25 mg) and tetracycline (30 μg) (Becton Dickinson).

Antibiotics tested in the present study were selected depending on their common use in veterinary practice and in hospitals. According to Van den Bogaard and Stobberingh (2000) and Galland *et al.* (2001) cephalothin, gentamicin, nitrofurantoin and norfloxacin are not approved for feedlot animals in contrast to ampicillin, amoxicillin and tetracycline. Furthermore, antibiotics used as growth promoters in veterinary practice in our region are mainly ampicillin, amoxicillin, tetracycline and sulfathiazole.

Disk diffusion susceptibility testing

Antibiotic susceptibility testing was determined by the agar diffusion method in accordance with NCCLS guidelines M23–A2 and M37–A2 (NCCLS 2001; NCCLS 2002; Miller *et al.* 2003), using *E. coli* NCTC 9001 as reference strain. Isolates were recorded as resistant to an antibiotic if growth was indistinguishable from that on a control plate without antibiotic.

Statistical analysis

Data were converted to binary code on the basis of sensitivity or resistance, and inter-isolate relationships were

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examined by CA applying average linkage method (Parveen *et al.* 1997). DA was performed (prior probabilities, equal; covariance matrix, pooled). The average rate of correct classification (ARCC) for each analysis was determined as described by Wiggins (1996). Relationships were also demonstrated with plot of principal component similarity coefficients. Computations were performed with SPSS, v.12.0 (SPSS Inc., Chicago, IL, USA).

Results

In total, 128 *E. coli* isolates, derived from human and animal faeces, showed variant resistance patterns to the 13 antibiotics tested. About 87·5% of all *E. coli* isolates (both human and animal) were resistant to one or more antibiotics. Human isolates showed multiple resistance ranging from two to all the antibiotics tested, while the resistance of animal isolates ranged from one to six antibiotics. In addition 26·7% of animal isolates were sensitive to all antibiotics. Isolates resistant to single antibiotics are shown in Table 2. Comparing human and animal isolates there was significant difference in levels of resistance to all antibiotics tested ($P < 0·05$). Without pooling all animal sources of *E. coli* strains together, goat isolates showed higher resistance when compared with cattle and sheep isolates, which was significantly different ($P < 0·05$) (Table 2).

Screening the resistance of *E. coli* isolates to multiple antibiotics the predominant MAR patterns of *E. coli* isolates are shown in Table 3. In general, human isolates showed higher resistance to combination of antibiotics than animal isolates. Specifically, 46 distinct resistance patterns were observed for human isolates compared with 24 patterns for animal isolates. The MAR indices for the isolates were determined according to Kaspar *et al.* (1990). The average MAR index for human isolates was 0·67 compared with 0·17 for animal isolates.

Performing CA, the relationships of antibiotic resistance patterns between human and animal *E. coli* isolates were based on coefficients of similarity measured by Squared Euclidean Distance (Fig. 1). The dendrogram was formed by clustering the 128 isolates into two major groups separating animal from human isolates. Cluster I contained the majority of animal isolates (90%) while

Table 2 Percentages of *Escherichia coli* human and animal isolates resistant to antibiotics

	% of resistant isolates				
Antibiotic	Human	Cattle	Sheep	Goat	Animal
Amikacin	89·7	ND	ND	ND	ND
Amoxicillin	86·7	ND	8·3	31·8	15
Ampicillin	89·7	7·14	16·7	36·4	21·7
Cephalothin	80·9	42·9	37·5	36·4	38·3
Chloramphenicol	50	ND	ND	ND	ND
Gentamicin	24·4	ND	ND	9·1	3·3
Nalidixic acid	55·9	ND	16·7	ND	6·7
Neomycin	64·7	ND	ND	59·1	21·7
Nitrofurantoin	41·2	ND	4·17	ND	1·7
Norfloxacin	45·6	ND	8·3	ND	3·3
Oxytetracycline	80·9	50	8·3	86·4	46·7
Sulfathiazole	67·6	ND	4·2	77·3	30
Tetracycline	80·9	3	ND	86·4	36·7

ND, none detected.

Table 3 Predominant antibiotic resistance patterns of *Escherichia coli* isolated from humans and animals

	% of isolates with indicated resistance pattern from source type	
Pattern of antibiotic resistance	Human	Animal
CEPH	ND	6·7
NOR	ND	3·3
OX	ND	5
CEPH-AMP	ND	5
CEPH-OX	1·5	3·3
CEPH-N-OX	1·5	ND
AN-AMP-AMX	2·9	ND
OX-TET-CEPH	ND	3·3
OX-TET-SU	ND	6·7
AMP-AMX-AN-CEPH	2·9	ND
OX-TET-SU-N	ND	6·7
OX-TET-AMP-AMX-AN	2·9	ND
OX-TET-AMP-AMX-CEPH	ND	3·3
OX-TET-SU-N-CEPH	1·5	3·3
OX-TET-SU-N-GM	ND	3·3
OX-TET-SU-N-AMP-AMX	ND	6·7
OX-TET-AN-AMP-AMX-C	1·5	ND
OX-TET-AN-AMP-AMX-CEPH	1·5	ND
N-AN-NAL-AMP-AMX-CEPH	1·5	ND
OX-TET-SU-AN-AMP-AMX-C	1·5	ND
OX-TET-N-AN-AMP-AMX-CEPH	1·5	ND
TET-SU-N-AN-AMP-AMX-CEPH	1·5	ND
OX-TET-SU-GM-N-AN-NAL-NOR-AMP-AMX-C-CEPH	10·3	ND
OX-TET-SU-N-AN-NAL-NOR-AMP-AMX-C-CEPH-NIT	17·6	ND
OX-TET-SU-GM-AN-NAL-NOR-AMP-AMX-C-CEPH-NIT	1·5	ND
OX-TET-SU-GM-N-AN-NAL-NOR-AMP-AMX-CEPH-NIT	1·5	ND
OX-TET-SU-GM-N-AN-NAL-NOR-AMP-AMX-C-NIT	1·5	ND
Resistance to all antibiotics	2·9	ND
Sensitivity to all antibiotics	ND	26·7

AN, amikacin; AMX, amoxicillin; AMP, ampicillin; CEPH, cephalothin; C, chloramphenicol; GM, gentamicin; NAL, nalidixic acid; N, neomycin; NIT, nitrofurantoin; NOR, norfloxacin; OX, oxytetracycline; SU, sulfathiazole; TET, tetracycline; ND, none detected.

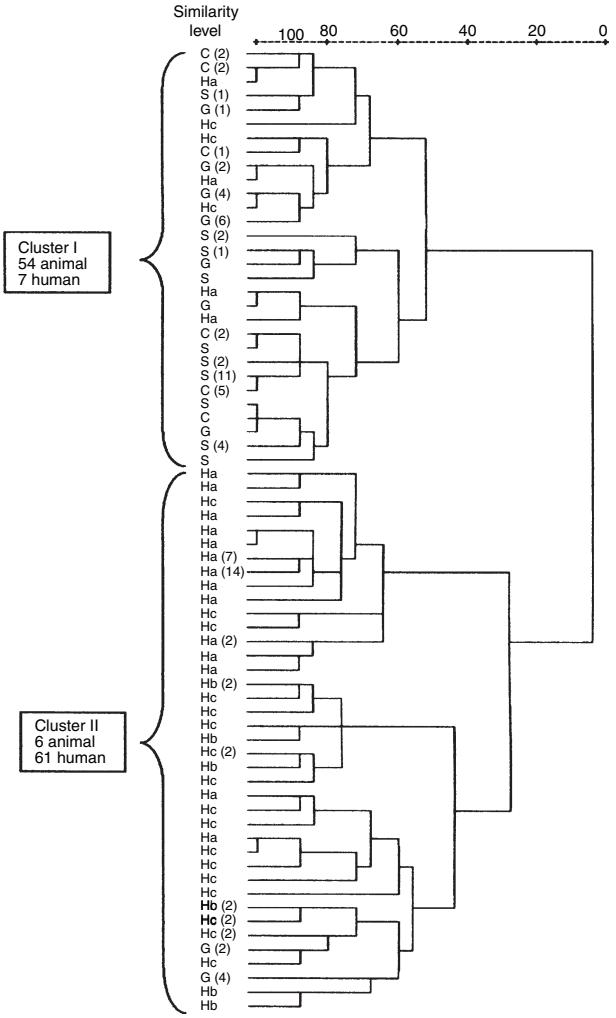

Figure 1 Dendrogram showing cluster formation of *Escherichia coli* isolates from humans and animals, using average linkage method. Prefixes indicate the source of isolates (C: cattle; G: goat; S: sheep; Ha: adult patient; Hb: healthy human; Hc: child patient) and number in parentheses is the number of isolates.

cluster II contained mainly human isolates (89·7%). The misclassifications of human *E. coli* isolates concerned four isolates from adult and three from paediatric patients. In addition, six animal isolates were classified along with the human ones and they were all goat isolates, which showed the highest resistance among animal *E. coli*. Generally, CA divided the isolates into the two large clusters, I and II, based on high (human isolates) and low (animal isolates) levels of antibiotic resistance. The presence of many subclusters indicates the prevalence of unique antibiotic resistance patterns among the isolates. Although cattle, sheep and goat isolates were not classified separately in distinct subclusters, they were pooled together in cluster I and were separated from human isolates of cluster II.

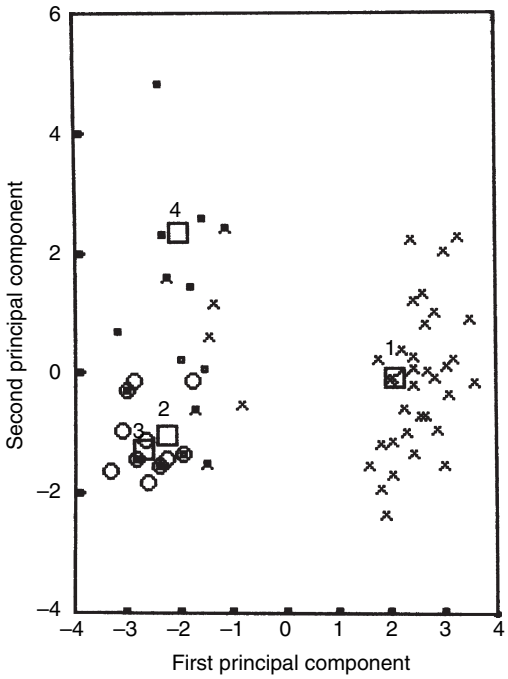

Fig. 2 Two-dimensional plot of antibiotic resistance patterns by principal component analysis. □: group centroids, ×: 1. human, □: 2. cattle, ○: 3. sheep, ■: 4. goat.

Similarly, human isolates were not distinguished among adult, children and isolates from healthy volunteers. Nevertheless, the majority of *E. coli* isolates from adult patients (94%) were all pooled in a group of cluster II, while the rest of human isolates along with six goat isolates were grouped in another subcluster (Fig. 1). Similar grouping was achieved by principal component analysis (Fig. 2) as human isolates were well distinguished from animal isolates with distinct group centroids.

Discriminant analysis was performed in order to validate the classification and separation of the 128 *E. coli* isolates of animal and human source. The sources of isolates (human, cattle, sheep and goat) were analysed based on resistance to the drugs tested. Cattle isolates were correctly classified much more poorly than human, sheep and goat isolates (Table 4). The ARCC using separate

Table 4 Discriminant analysis of antibiotic resistance profiles of *Escherichia coli* isolated from various sources*

	% of isolates classified as				
Source (no. of isolates)	Human	Cattle	Sheep	Goat	Total
Human (68)	89·7	2·9	1·5	5·9	100
Cattle (14)	0	50·0	50·0	0	100
Sheep (24)	0	8·3	91·7	0	100
Goat (22)	0	0	13·6	86·4	100

*The average rate of correct classification for this analysis was 85·2%.

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Table 5 Discriminant analysis of antibiotic resistance profiles of *Escherichia coli* isolated from animals and humans*

Source (no. of isolates)	% of isolates classified as		
	Animal	Human	Total
Animal (60)	98·4	1·6	100
Human (68)	0	100	100

*The average rate of correct classification for this analysis was 99·2%.

sources was 85·2%. Since the most important goal was to differentiate between animal and human sources of pollution, pooling all animal *E. coli* isolates in one group and human *E. coli* isolates in another, the ARCC was improved to a level of 99.2% (Table 5).

Discussion

The present study focused on determining antibiotic resistance patterns of *E. coli* isolates derived from human and animal faecal material and differentiating them according to their source. The selection of a sufficient number of antibiotics commonly used in veterinary practice and hospitals, provided data that placed the isolates into major groups consisting of human and animal *E. coli* separately, with high levels of specificity.

Human isolates showed high resistance to combinations of antibiotics, regardless of their specific origin (adults and children hospitalized, healthy volunteers) with an average MAR index (0·67) 3·9 times greater than the average MAR index of animal isolates (0·17), which were more sensitive to single and multiple drugs. This happened mainly due to the excess use of antibiotics especially in human treatment. Similar results were obtained from other studies (Parveen *et al.* 1997, 1999, Guan *et al.* 2002).

Among the animal isolates, the isolates derived from goats showed the highest rates of resistance while isolates from cattle had the lowest. These results were in compliance with other studies (Parveen *et al.* 1999; Guan *et al.* 2002). Furthermore, animal isolates resistant to antibiotics not suitable for veterinary practice were low except for cephalothin, to which 80·9% of animal isolates were resistant, probably because of cross-resistance (Van den Bogaard and Stobberingh 2000). Percentages of isolates resistant to ampicillin, amoxicillin and tetracycline (Table 2), indicate their extensive use in animals.

Among the 128 *E. coli* isolates there was a considerable variance of resistance profiles (70 in total). Human isolates shared 46 patterns compared with 24 of animal isolates. The greater variance among human isolates was obvious in the dendrogram constructed by CA (Fig. 1) and in the formation of subclusters. The variety of resistance profiles may come as a consequence of the randomness of the strain collection. The fact that *E. coli* isolates were obtained from quite an expanded region and from random hosts indicates that they were imposed to different selective pressure, which resulted in the development of different profiles of antibiotic resistance.

Both CA and DA were suitable statistical procedures for analysing antibiotic resistance patterns. Each procedure could be used alone but their combination has the advantage of an additional confidence, especially when the two methods provide the same answers. The dendrogram formed by CA shows the level of separation by source that occurred and the relatedness of the isolates tested. DA provides the percentages of isolates from different sources and the ARCC, which can easily be determined (Tables 4 and 5). The different resistance patterns among the isolates resulted in their separation and grouping in two major distinct clusters with a dissimilarity level of 73% (Fig. 1). Similar results were obtained by principal component analysis which divided isolates into two distinct groups with few exceptions of misclassification (Fig. 2). DA validated this classification, estimating the ARCC according to the source of the micro-organisms tested. What is important for public health is to distinguish animal from human isolates; thus all animal sources were pooled together, providing even better ARCC (99·2%) than that of separated sources into humans, cattle, sheep and goats (Tables 4 and 5). The high ARCC indicates the satisfactory discrimination and classification of *E. coli* isolates applying MAR with the specific antibiotics.

The main disadvantage of MAR analysis is the stable patterns of antibiotic resistance of bacteria, as antibiotic use varies significantly (Harwood *et al.* 2000). Considering the fact that the selective pressure of antibiotic treatment on the commensal microflora forms differentiate the resistance profiles (Oppegaard *et al.* 2001), the databases developed for DA require periodic updating. Furthermore, as there are few techniques that overcome the drawbacks of MAR analysis (Galland *et al.* 2001) it is important to combine these phenotypic results with the fingerprints of the isolates obtained from a DNA genotyping method such as RAPD-PCR (data not shown) based on the statistical analysis of fingerprint differences. Nevertheless, the determination of antibiotic resistance profiles of *E. coli* is often preferred as a simple, direct and cost-effective method.

In the present study there was an effort to test as many drugs used in humans and animals as possible. It seems that the more drugs that are used the better the chances of getting a combination that is successful in discriminating among a particular set of isolates (Wiggins 1996; Wiggins *et al.* 1999). The number of drugs tested (13) is considered satisfactory for valid and reliable results.

Inevitably, reducing their number may result in altering the outline of CA and in lowering the ARCC of DA. Analysis proposed by Wiggins (1996) using five antibiotics did not provide adequate separation of isolates from known sources, showing that it is necessary to test a wider range of drugs. Other studies with satisfactory results used nine (Harwood *et al.* 2000), 10 (Khan *et al.* 2002), 11 (Kaspar *et al.* 1990) and 13 (Hagedorn *et al.* 1999) different antibiotics, verifying the need to test a variety of drugs in order to accomplish a high level separation of the isolates. In the present study a minimal drug set of nine antibiotics provided the clustering as outlined but with a lower level of separation and ARCC.

Finally, the randomness of the strain collection combined with the results derived from the statistical evaluation support our proposal of applying MAR analysis as a useful tool for differentiating between human and animal source of *E. coli* isolates. Recognizing that a specific *E. coli* MAR profile may not always correlate with human or animal pollution it is necessary to further examine the isolates with other methods to develop reliable associations between MAR profiles and sources of pollution.

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