MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE IN *ESCHERICHIA COLI* STRAINS FROM A DAIRY CATTLE FARM AND ITS SURROUNDINGS

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Running title: Antimicrobial-resistant *E. coli* from a dairy cattle farm and its surroundings

ABSTRACT

BACKGROUND: This study describes the phenotypic and genotypic characteristics of 78 genetically different *Escherichia coli* recovered from the air and exudate samples of a dairy cattle farm and its surroundings in Spain, in order to gain insight into the flow of antimicrobial resistance through the environment and food supply.

RESULTS: Antimicrobial resistance was detected in 21.8% of the 78 *E. coli* isolates analyzed (resistance for at least one of the 14 agents tested). The highest resistance rates were recorded for ampicillin, nalidixic acid, trimethoprim/sulfamethoxazole and tetracycline. The resistance genes detected were as follows [antibiotic (number of resistant strains), gene (number of strains)]: ampicillin (9), $bla_{\text{TEM-1}}$(6); tetracycline (15), *tet*(A) (7), *tet*(B) (4), *tet*(A)+ *tet*(B) (1); chloramphenicol (5), *cmlA* (2), *floR*(2); trimethoprim/sulfamethoxazole (10), *sul2* (4), *sul1* (3), *sul3* (2), *sul1*+ *sul2* (1); gentamicin-tobramycin (1), *ant(2'')* (1). About 14% of strains showed a multidrug-resistant phenotype and, of them, 7 strains carried class 1 integrons containing predominantly the *dfrA1-aadA1* array. One multidrug-resistant strain was found in both inside and outside air, suggesting that the airborne spread of multidrug-resistant bacteria from the animal housing facilities to the surroundings is feasible.

CONCLUSIONS: This study gives a genetic background of the antimicrobial resistance problem in a dairy cattle farm and shows that air can act as a source for dissemination of antimicrobial-resistant bacteria.

Keywords: resistance genes, air, cattle, farm environment

INTRODUCTION

The spread of resistant bacteria as well as antimicrobial resistance genes is a global problem, being present in many environments, all of them interconnected by different paths. For instance, in recent years, multidrug-resistant bacteria (including extended-spectrum beta-lactamase – ESBL-producing strains) have been detected in vegetable food which could potentially be transmitted to humans through the food chain[1,2]. However, there is still lack of data about emission sources of these bacteria. In a previous study carried out by our group[3], in order to demonstrate the importance of the air as a vehicle for *E. coli* dissemination, air samples from inside of a dairy cattle farm and the immediate surroundings of cultivated fields were studied by pulsed-field gel electrophoresis (PFGE). The comparison of genetic profiles suggested that the strains isolated from inside and outside the farm were related, leading to the conclusion that airborne transfer of *E. coli* from inside the cattle farm to surrounding crops areas was feasible. Focusing on antimicrobial resistant bacteria, findings from a study performed in broiler chicken fattening farms and their environment revealed a potential airborne exchange of ESBL-producing *E. coli*[4]. Similar evidences, suggesting the spread of antibiotic resistance genes from beef cattle feed yards to the environment through aerial transport, have been recently reported by other authors in a research conducted in USA[5]. This last study was performed using quantitative PCR-based tools giving a general idea of the concentrations of antibiotic resistance gene pool in the total microbial community DNA from air samples. However, it was not designed to analyze the viability of the bacteria harboring these antimicrobial determinants after aerial transport. In the present work carried out in Spain, we isolate, phenotypically characterize and molecular analyze the resistance genotype and integron content of culturable *E. coli* strains previously recovered from air and exudate samples of a dairy cattle farm and its surroundings[3], to gain insight into the flow of antimicrobial resistance through the environment and food supply.

EXPERIMENTAL

Bacterial strains included in the study.

One-hundred-seventeen *E. coli* isolates were recovered in a previous study[3] from organic exudates (42 isolates) and from samples of inside and outside air (75 isolates) of a cattle farm. These isolates were recovered from air samples taken in different points inside of the cattle farm and from immediate outside surroundings (at distance of 50, 100 and 150 m in four directions) and from organic exudates (dirty straw and manure inside the dairy farm). The initial isolation and enumeration of *E. coli* isolates was performed in Chromocult Coliform Agar (Merck) and they were identified by biochemical and molecular methods[3]. Seventy-eight different PFGE profiles were detected among the 117 *E. coli* isolates. One *E. coli* strain from each of the 78 different PFGE profiles was selected (55 of air, 23 of organic exudates) and included in the present study, making a collection of 78 *E. coli* strains that has been used in this study for characterization of antimicrobial resistance phenotype and genotype.

Antimicrobial susceptibility testing and resistance genes

Antimicrobial susceptibility testing was performed by the disc diffusion method, according to the Clinical Laboratory Standards Institute guidelines[6]. The susceptibility of the *E. coli* isolates was tested for 14 antimicrobial agents commonly used against *E. coli* infections in humans: ampicillin, amoxicillin/clavulanate, ceftazidime, ceftriaxone, cefoxitin, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, chloramphenicol, trimethoprim/sulfamethoxazole and tetracycline. *E. coli* ATCC 25922 was used as a control strain.

Phenotypically resistant *E. coli* isolates were characterized at the molecular level for their antimicrobial resistance mechanisms. Simplex PCR and subsequent sequencing were used for the detection of drug-resistant genes associated with beta-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{PSE}, and $bla_{\text{CTX-M}}$). Mutations in the chromosomal *amp*C promoter region were also determined by PCR and sequencing. Amino acid changes in GyrA and ParC proteins were studied by PCR and sequencing of the corresponding genes in fluoroquinolone-resistant isolates. In addition, plasmid-mediated quinolone-resistance genes (PMQR) (*qnrA*, *qnrB*, *qnrS*,

aac(6´)-Ib-cr, *qepA*), as well as aminoglycoside (*aac (3)-I, aac (3)-II, aac (3)-III, aac (3)-IV, ant*(2"), *aac(6´)-Ib*), sulfonamide (*sul1*, *sul2*, *sul3*), chloramphenicol (*cmlA*, *florR*, *catB*) and tetracycline resistance genes (*tet*(A), *tet*(B)) were tested by PCR and sequencing[7].

The presence of integrons was detected by PCR amplification of the integrase gene *intI1* (for class 1 integrons) and *intI2* (for class 2 integrons). To characterize their genetic structure, PCR "primer-walking" strategy was used in order to get the complete gene cassette arrangement[7].

RESULTS AND DISCUSSION

The rate of antimicrobial resistance in the collection of 78 genetically different *E. coli* strains recovered from air samples and organic exudates of a cattle farm was 21.8% (17 out of 78 strains showed resistance to at least one of the 14 antimicrobial agents tested). The highest resistance rates were recorded for ampicillin, nalidixic acid, trimethoprim/sulfamethoxazole and tetracycline (11.5-17.9%).These antimicrobial agents are frequently used both in clinical and veterinary practice. No ESBL-producing *E. coli* were found in the studied *E. coli* collection. It is remarkable the detection of eleven strains (14.1%) which showed a multidrug-resistant (MDR) phenotype, including at least 3 families of antibiotics. Co-resistance to ampicillin, tetracycline, trimethoprim/sulfamethoxazole and nalidixic acid was observed in most of the multi-resistant isolates. The presence of integrons appears to be associated with these MDR phenotypes, as suggested by the fact that 7 out of 11 MDR strains carried class 1 integrons (Table 1). About 57% contained the conserved *qacE*Δ*1-sul1* region and, in all of them, the array *dfrA1-aadA1* was identified. The presence of classic class 1 integrons containing trimethoprim (*dfr*) and streptomycin (*aad*) resistance encoding genes have been frequently reported in *E. coli* isolates recovered from different sources, including food producing animals[8,9], humans[7] and the environment[1].

The moderate percentage of bacteria resistant to antimicrobials found in the present work is in accordance with other studies carried out in cattle farms[10], which shows lower resistance rates than those focused on other livestock animals, such as poultry or pigs[8, 11-14]. This fact reflects variations in the use of antimicrobial agents among the different livestock production sectors, as

have been reported in a previous study[15].It should be noted that the percentage of resistance shown in this work was calculated among the clonally unrelated *E. coli* strains, so there might be variations depending on the frequency of certain clones.

Molecular analysis showed the following acquired resistance genes among the studied *E. coli* strains [antibiotic (number of resistant strains)/gene (number of strains)] (Table 1): Ampicillin (9) / $bla_{\text{TEM-1}}$(6); tetracycline (15) / *tet*(A)(7)/*tet*(B) (4)/*tet*(A) + *tet*(B)(1); chloramphenicol (5) / *cmlA* (2)/*floR*(2); trimethoprim/sulfamethoxazole (10) / *sul1* + *sul2* (1)/ *sul2* (4)/*sul1* (3)/*sul3* (2); and gentamicin-tobramycin (1) / *ant(2'')* (1). Resistance to fluoroquinolones was due to amino acid changes at positions 83 (S83L) and 87 (D87N) of the GyrA protein and at position 80 of the ParC protein [substitutions at this position were serine for isoleucine in two isolates (ZO10, ZO59) and serine for arginine in the other two ones (ESTE50, ESTE51)].In four cases (three for ampicillin and one for chloramphenicol), the genes responsible for resistance could not be identified, suggesting other possible resistance mechanisms. It is remarkable the detection of the *ant(2'')* gene associated with resistance to gentamicin and tobramycin in one of the strains. This gene, which encodes a 2"-O-adenyltransferase aminoglycoside-modifying enzyme, is infrequently identified in non-human resistant *E. coli* isolates according to a previous microarray based comparative study on gentamicin resistant strains from food producing animals and humans[16]. With regard to chloramphenicol resistant strains, it is noteworthy the detection of the *floR* and *cmlA* genes in the same percentage. Unlike *floR*, *cmlA* marker is not very common among *E. coli* of bovine origin[9, 16]. The type and distribution of the ampicillin ($bla_{\text{TEM-1}}$) and tetracycline [*tet*(A), *tet*(B)] resistance determinants shown in this study are in agreement with the data reported in a collection of MDR *E. coli* strains recovered from cattle and the farm environment in Ireland[9].

It is of interest to highlight that one MDR strain (ESTE50), which showed resistance to chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline and quinolones, was found in both inside and outside air of the dairy cattle farm. This result demonstrates that the airborne spread of MDR bacteria from cattle farms to the immediate environment is feasible. Similar findings carried out in a broiler chicken farm have been recently reported by other authors[4]. The

fact that the farm studied in the present study was bordered by agricultural fields could pose a risk of crops contamination and, consequently, led to antibiotic-resistant bacteria entering the food chain. However, the risk for human health cannot be estimated with available data and further studied are needed.

Supporting the findings of very recent studies[4, 5], this work reveals that the air seems to be an important vehicle for the transference of bacteria and their resistance genes from the farm field to the external environment. Both commensal and pathogenic bacteria as well as susceptible or resistant bacteria can be disseminated through this way, highlighting the complexity of routes of dispersion of the microorganisms in the different environments.

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Table 1. Phenotypic and genotypic characteristics of antimicrobial resistant *E.coli* detected in air or exudates collected from inside and outside the cattle farm.

Strain	Origin	Resistance phenotype[b]	Resistance genes outside the integron	Amino acid changes		Class 1 integron	
				GyrA	ParC	Int1/3'-CS	Integron structure
P8	Organic exudate (inside)	AMP, SXT, TE, NA	*sul1*, *tet*(A)	ND[c]	ND	+/+	*dfrA1-aadA1*
P20	Air (inside)	SXT, TE, NA	*sul1*, *tet*(A)	ND	ND	+/+	*dfrA1-aadA1*
ESTE42	Air, East, 50 m (outside)	AMP	bla_{TEM-1}	ND	ND	-	-
ESTE50[a]	Air, East, 100 m (outside) / Air (inside)	C, SXT, TE, NA, CIP	*sul3*, *cmlA*	S83L, D87N	S80R	+/-	NI[d]
ESTE51	Air, East, 100 m (outside)	C, SXT, TE, NA, CIP	*sul3*, *cmlA*	S83L, D87N	S80R	-	-
ZO6	Air (inside)	AMP, AMC, SXT, TE	*sul2*, *tet*(A)	ND	ND	-	-
ZO08	Air (inside)	C, SXT, TE, NA, GN, TB	*sul2*, *tet*(B), *ant*(2")	ND	ND	+/-	NI
ZO10	Air (inside)	AMP, C, SXT, TE, NA, CIP	bla_{TEM-1}, *floR*, *sul2*, *tet*(A), *tet*(B)	S83L, D87N	S80I	+/-	NI
ZO20	Air (inside)	TE	*tet*(B)	ND	ND	-	-
ZO22	Air (inside)	AMP, SXT, TE	bla_{TEM-1}, *sul1*, *sul2*, *tet*(A)	ND	ND	+/+	*dfrA1-aadA1*
ZO59	Air (inside)	AMP, SXT, TE, NA, CIP	bla_{TEM-1}, *sul1*, *tet*(A)	S83L, D87N	S80I	+/+	*dfrA1-aadA1*
E1	Organic exudate (inside)	TE	*tet*(B)	ND	ND	-	-
E6	Organic exudate (inside)	AMP, SXT, TE, NA	bla_{TEM-1}, *sul2*	ND	ND	-	-
E12	Organic exudate (inside)	AMP, C, TE	*floR*, *tet*(A)	ND	ND	-	-
E15	Organic exudate (inside)	TE, NA	*tet*(A)	ND	ND	-	-
E18	Organic exudate (inside)	AMP, TE	bla_{TEM-1}, *tet*(B)	ND	ND	-	-
E20	Organic exudate (inside)	NA	-	ND	ND	-	-

[a]This strain was isolated both inside and outside the dairy cattle farm.

[b]AMP: ampicillin; AMC: amoxicillin/clavulanate; C: chloramphenicol; SXT: trimethoprim/sulfamethoxazole; TE: tetracycline; NA: nalidix acid; CIP: ciprofloxacin; GN: gentamicin; TB: tobramycin.

[c] ND: Non determined.

[d] NI: Non identified array with performed PCRs.