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High Diversity of Extended-Spectrum β-Lactamases in *Escherichia coli* Isolates from Italian Broiler Flocks[▽]

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We characterized 67 *Escherichia coli* isolates with reduced susceptibility to cefotaxime or ceftiofur obtained from healthy broilers housed in five Italian farms. The $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}32}$ and $bla_{SHV\text{-}12}$ β-lactamase genes were identified on IncI1, IncN, or IncFIB plasmids. Considerable genetic diversity was detected among the extended-spectrum β-lactamase (ESBL)-producing isolates, and we identified indistinguishable strains in unrelated farms and indistinguishable plasmids in genetically unrelated strains. The detection of highly mobile plasmids suggests a potential animal reservoir for β-lactamase genes.

Escherichia coli resistant to extended-spectrum cephalosporins through the production of extended-spectrum β-lactamases (ESBLs) is isolated at an increasing rate among different hosts and countries, thus representing a potential public health risk (2, 3). Typically, ESBLs are borne on mobile genetic elements and can therefore spread both clonally and horizontally among different lineages of bacteria (5).

To determine the role of animals as potential reservoirs for ESBL-producing bacteria, there is a need for improved knowledge of the factors that promote dissemination of resistance determinants, including the role of the intestinal flora (4). In this study, we characterized a collection of 67 *E. coli* isolates showing reduced susceptibility to cefotaxime (MIC $\geq$ 2 μg/ml) or ceftiofur (MIC $\geq$ 8 μg/ml). The isolates were obtained during July 2007 by processing fecal samples collected by the "sock method" (1, 22) in a broiler-parent farm and four broiler farms located in northeastern Italy. The farms were ordinary with respect to farm management and biosecurity, thus implying that no contact among the animals reared in different farms could have taken place. One flock (i.e., a group of chickens sharing the same airspace) per farm was sampled (Table 1). All broiler flocks (here identified as flocks 1, 2, 4, and 5) hatched from eggs incubated in the same hatchery, and the parent flock (here identified as flock 6) was among the flocks that provided eggs to that hatchery. Indication of ESBL presence resulted from the ESBL phenotypic detection test performed with the disk method (7, 10). Amplification of the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes was done by PCR using primers and conditions previously described (15, 16) on whole-genome bacterial DNA (GenElute bacterial genomic DNA kit; Sigma-Aldrich, Brøndby, Denmark). PCR products were purified (exonuclease I, *E. coli*, and FastAP thermosensitive alkaline phosphatase; Fermentas, Helsingborg, Sweden) and sequenced (Macrogen Inc., Seoul, South Korea). Comparison of the nucleotide sequences and the derived amino acid sequences with previously described sequences (GenBank database http://www.ncbi.nlm.nih.gov/ and www.lahey.org/studies/webt.html, respectively) showed that the $bla_{TEM\text{-}1}$, $bla_{SHV\text{-}12}$, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}32}$ genes were present in this collection (Fig. 1). The genetic relatedness of the *E. coli* isolates was determined by amplified fragment length polymorphism (AFLP) analysis as previously described (1, 6). Using a similarity cutoff value of

TABLE 1. Anamnestic information about the flocks sampled

Flock	Type of animals	Age (days)	Antimicrobial treatment history	Antimicrobial treatment history of the preceding flock	No. of isolates included in the study
1	Broilers	35	None	Oxytetracycline, sulfadiazine-trimethoprim	19
2	Broilers	12	Amoxicillin	Flumequine, amoxicillin, sulfadiazine-trimethoprim, colistin	19
4	Broilers	70	Enrofloxacin, tylosin	None	14
5	Broilers	35	Amoxicillin	Amoxicillin	5
6	Parents	240	Tylosin, tetracycline, colistin	Tylosin, tetracycline, colistin	10

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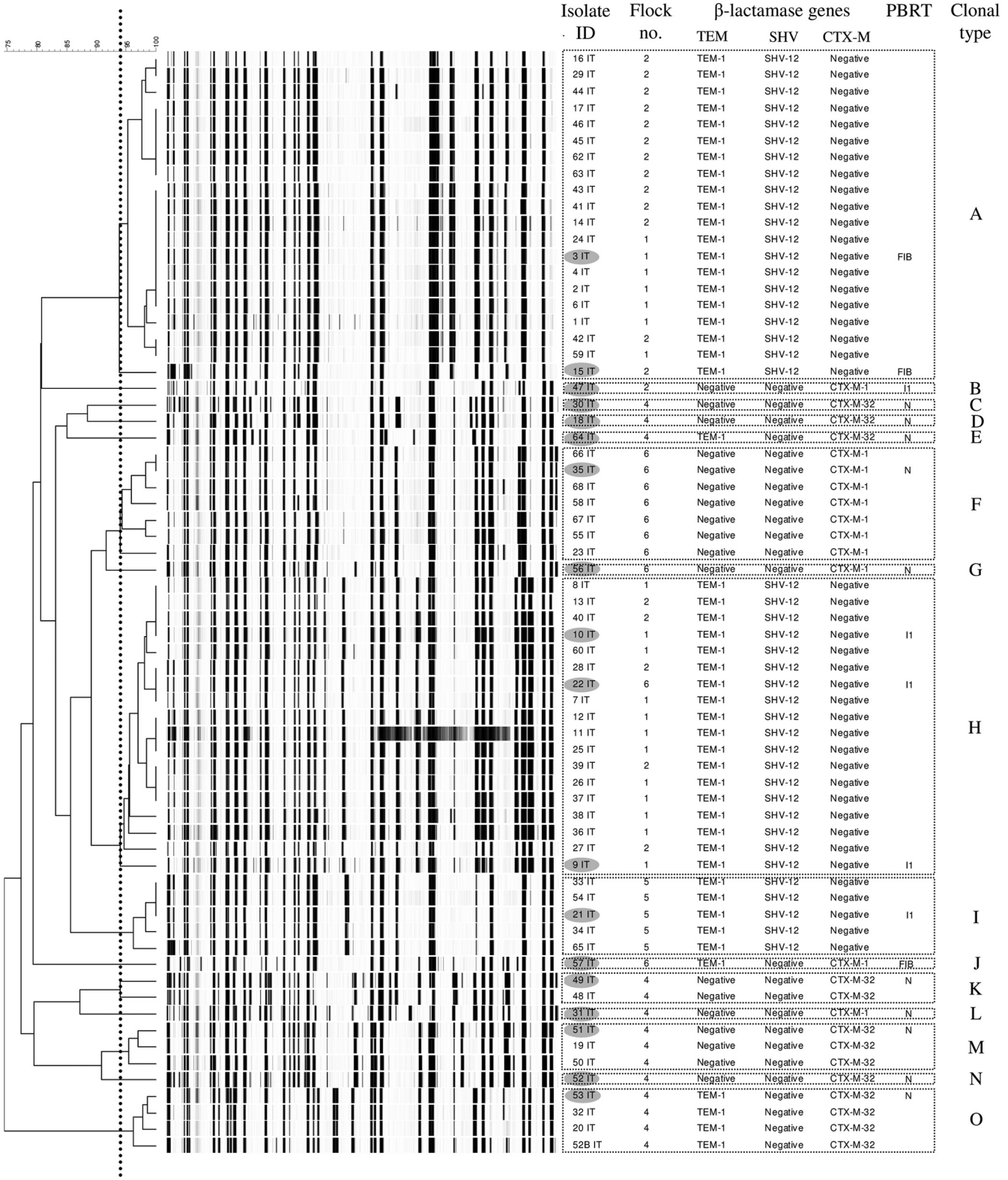

Isolate ID	Flock no.	β-lactamase genes			PBRT	Clonal type
		TEM	SHV	CTX-M		
16 IT	2	TEM-1	SHV-12	Negative		A
29 IT	2	TEM-1	SHV-12	Negative		
44 IT	2	TEM-1	SHV-12	Negative		
17 IT	2	TEM-1	SHV-12	Negative		
46 IT	2	TEM-1	SHV-12	Negative		
45 IT	2	TEM-1	SHV-12	Negative		
62 IT	2	TEM-1	SHV-12	Negative		
63 IT	2	TEM-1	SHV-12	Negative		
43 IT	2	TEM-1	SHV-12	Negative		
41 IT	2	TEM-1	SHV-12	Negative		
14 IT	2	TEM-1	SHV-12	Negative		
24 IT	1	TEM-1	SHV-12	Negative		
3 IT	1	TEM-1	SHV-12	Negative	FIB	
4 IT	1	TEM-1	SHV-12	Negative		
2 IT	1	TEM-1	SHV-12	Negative		
6 IT	1	TEM-1	SHV-12	Negative		
1 IT	1	TEM-1	SHV-12	Negative		
42 IT	2	TEM-1	SHV-12	Negative		
59 IT	1	TEM-1	SHV-12	Negative		
15 IT	2	TEM-1	SHV-12	Negative	FIB	
47 IT	2	Negative	Negative	CTX-M-1	I1	B
30 IT	4	Negative	Negative	CTX-M-32	N	C
18 IT	4	Negative	Negative	CTX-M-32	N	D
64 IT	4	TEM-1	Negative	CTX-M-32	N	E
66 IT	6	Negative	Negative	CTX-M-1		
35 IT	6	Negative	Negative	CTX-M-1	N	
68 IT	6	Negative	Negative	CTX-M-1		
58 IT	6	Negative	Negative	CTX-M-1		F
67 IT	6	Negative	Negative	CTX-M-1		
55 IT	6	Negative	Negative	CTX-M-1		
23 IT	6	Negative	Negative	CTX-M-1		
56 IT	6	Negative	Negative	CTX-M-1	N	G
8 IT	1	TEM-1	SHV-12	Negative		
13 IT	2	TEM-1	SHV-12	Negative		
40 IT	2	TEM-1	SHV-12	Negative		
10 IT	1	TEM-1	SHV-12	Negative	I1	
60 IT	1	TEM-1	SHV-12	Negative		
28 IT	2	TEM-1	SHV-12	Negative		
22 IT	6	TEM-1	SHV-12	Negative	I1	
7 IT	1	TEM-1	SHV-12	Negative		
12 IT	1	TEM-1	SHV-12	Negative		
11 IT	1	TEM-1	SHV-12	Negative		H
25 IT	1	TEM-1	SHV-12	Negative		
39 IT	2	TEM-1	SHV-12	Negative		
26 IT	1	TEM-1	SHV-12	Negative		
37 IT	1	TEM-1	SHV-12	Negative		
38 IT	1	TEM-1	SHV-12	Negative		
36 IT	1	TEM-1	SHV-12	Negative		
27 IT	2	TEM-1	SHV-12	Negative		
9 IT	1	TEM-1	SHV-12	Negative	I1	
33 IT	5	TEM-1	SHV-12	Negative		
54 IT	5	TEM-1	SHV-12	Negative		
21 IT	5	TEM-1	SHV-12	Negative	I1	I
34 IT	5	TEM-1	SHV-12	Negative		
65 IT	5	TEM-1	SHV-12	Negative		
57 IT	6	TEM-1	Negative	CTX-M-1	FIB	J
49 IT	4	Negative	Negative	CTX-M-32	N	K
48 IT	4	Negative	Negative	CTX-M-32		
31 IT	4	Negative	Negative	CTX-M-1	N	L
51 IT	4	Negative	Negative	CTX-M-32	N	
19 IT	4	Negative	Negative	CTX-M-32		M
50 IT	4	Negative	Negative	CTX-M-32		
52 IT	4	Negative	Negative	CTX-M-32	N	N
53 IT	4	TEM-1	Negative	CTX-M-32	N	
32 IT	4	TEM-1	Negative	CTX-M-32		O
20 IT	4	TEM-1	Negative	CTX-M-32		
52B IT	4	TEM-1	Negative	CTX-M-32		

FIG. 1. Dendrogram showing the genotypic relatedness of 67 *Escherichia coli* isolates based on AFLP fingerprints. A Dice coefficient and the unweighted-pair group method using average linkages (UPGMA) algorithm were used with a position tolerance of 1%. The scale bar represents the percentage of similarity, and the vertical, dotted line indicates the cutoff value for identifying clonal types. PBRT, plasmid-based replicon typing as determined with a subset of isolates indicated by gray-shaded ovals.

TABLE 2. Genetic and phenotypic traits of ESBL-producing *Escherichia coli* from broiler flocks

Flock	Isolate	AFLP clonal type (no. of strains within clonal type)	β-Lactamase gene(s)[a]	ClaI plasmid RFLP profile	PstI plasmid RFLP profile	Replicon typing	Additional resistances[b]	*sul* genes[c]
1	3	A (20)	TEM-1, SHV-12	9	l	FIB	TET, SUL, TMP, FFC, CHL, NAL, CIP, STR	*sul1*, *sul3*
1	9	H (18)	TEM-1, SHV-12	3	c	I1	TET, SUL, TMP, FFC, CHL, NAL, STR	*sul2*, *sul3*
1	10	H (18)	TEM-1, SHV-12	11	n	I1	TET, SUL, TMP, NAL, STR	*sul1*, *sul2*
2	15	A (20)	TEM-1, SHV-12	9	l	FIB	TET, SUL, TMP, FFC, CHL, NAL, CIP, STR	*sul1*, *sul3*
2	47	B (1)	CTX-M-1	8	i	I1	TET, SUL, TMP, NAL	*sul1*, *sul3*
4	18	D (1)	CTX-M-32	5	f	N	TET, NAL	
4	30	C (1)	CTX-M-32	5	f	N	TET, NAL	
4	31	L (1)	CTX-M-1	2	b	N	SUL, NAL, CIP, STR	*sul1*
4	49	K (2)	CTX-M-32	6	g	N	TET, SUL, NAL, CIP, SPT	*sul2*
4	51	M (3)	CTX-M-32	4	d	N	TET, SUL, TMP, CHL, NAL, CIP, STR	*sul2*
4	52	N (1)	CTX-M-32	5	f	N	TET, SUL, TMP, CHL, NAL, CIP, STR	*sul1*, *sul2*
4	53	O (4)	TEM-1, CTX-M-32	5	f	N	TET, SUL, TMP, CHL, NAL, CIP, STR, GEN	*sul1*, *sul2*, *sul3*
4	64	E (1)	TEM-1, CTX-M-32	5	f	N	TET, SUL, CHL, NAL, CIP	*sul3*
5	21	I (5)	TEM-1, SHV-12	1	a	I1	TET, SUL, TMP, FFC, CHL, STR	*sul1*, *sul2*, *sul3*
6	22	H (18)	TEM-1, SHV-12	7	h	I1	TET, SUL, TMP, FFC, CHL, NAL, STR	*sul1*, *sul2*, *sul3*
6	35	F (7)	CTX-M-1	5	e	N	TET	
6	56	G (1)	CTX-M-1	5	e	N	TET	
6	57	J (1)	TEM-1, CTX-M-1	10	m	FIB	TET, SUL, CHL, NAL	*sul1*, *sul3*

[a] β-Lactamase genes were determined by PCR and by sequencing in the donors. Genes transferred by transformation, as determined by PCR but not by sequencing, are underlined.
[b] CHL, chloramphenicol; CIP, ciprofloxacin; FFC, florfenicol; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfonamides; TET, tetracycline; TMP, trimethoprim. Patterns transferred by transformation are underlined.
[c] Genes transferred by transformation are underlined.

≥94% (1), 15 different clonal types were identified (A to O in Fig. 1). Eighteen strains were selected for plasmid analysis by selecting one strain per clonal type from each flock. Within one AFLP type, however, two strains from the same flock were examined to determine the intraclonal diversity. Plasmid DNA (GeneJET plasmid miniprep kit; Fermentas, Helsingborg, Sweden) was used to transform electrocompetent Genehog *E. coli* (Invitrogen, Taastrup, Denmark). Transformants were selected on brain heart infusion (BHI) agar (Merck, Glostrup, Denmark) plates containing 2 μg/ml of cefotaxime and tested by colony PCR (15, 16) to confirm the presence of ESBL-encoding genes. Plasmid DNA (GeneJET plasmid miniprep kit; Fermentas, Helsingborg, Sweden) from the transformants was analyzed by restriction fragment length polymorphism (RFLP) analysis and PCR-based replicon typing (PBRT) (3). IncN ($n = 10$), IncI1 ($n = 5$), or IncFIB ($n = 3$) plasmids were identified (Fig. 1 and Table 2). Twelve different plasmid profiles were visualized on a 0.8% agarose gel after enzymatic digestion with ClaI and PstI (FastDigest; Fermentas, Helsingborg, Sweden). Notably, indistinguishable band patterns were obtained in (i) two $bla_{\text{CTX-M-1}}$ IncN plasmids found in two closely related *E. coli* strains (with 91.6% similarity according to the analysis of the AFLP patterns) from flock 6, (ii) five $bla_{\text{CTX-M-32}}$ IncN plasmids found in five unrelated *E. coli* strains from flock 4, and (iii) two $bla_{\text{SHV-12}}$ IncFIB plasmids found in two clonally related *E. coli* strains from flocks 1 and 2 (Fig. 1 and Table 2). The disk diffusion test (9, 10) showed that all donors were resistant to at least two additional antimicrobial classes (Table 2). Seventeen (94%) isolates were resistant to tetracycline, and 15 (83%) isolates were resistant to nalidixic acid, 8 (44%) of which were ciprofloxacin resistant. Sulfonamide resistance alone or in combination with trimethoprim resistance was detected in 4 (22%) and 10 (55%) donor isolates, respectively. No resistance against amikacin, colistin, and imipenem was detected. Sulfonamide resistance occurred in seven (39%) transformants and was associated with trimethoprim resistance in one (5%) case (Table 2). The cotransfer of *sul2* ($n = 1$), *sul3* ($n = 5$), and *sul1* and *sul3* ($n = 1$) genes was determined in such transformants by the use of PCR as previously described (Table 2) (14). As measured by agar dilution (8), the MIC of ciprofloxacin for all the transformants was <0.03 μg/ml, indicating that possible *qnr*-mediated resistance to this fluoroquinolone was not cotransferred by the ESBL-producing plasmids.

We isolated ESBL-producing *E. coli* in flocks that did not receive treatments with cephalosporins (Table 1). Treatments with other antimicrobials (Table 1) could have coselected for the bacteria described. Although a limited number of flocks and bacterial isolates was included in the study, the genetic environment for the ESBL-encoding genes was highly variable. The $bla_{\text{SHV-12}}$ gene was associated with three major clones (A, H, and I) detected in four flocks. These strains carried IncI1 (H

and I) or IncFIB (A) plasmids. The $bla_{CTX\text{-}M\text{-}1}$ gene and the closely related $bla_{CTX\text{-}M\text{-}32}$ gene were mainly associated with IncN plasmids found in 10 different clonal types in three flocks. As clearly shown in flock 4 (Table 2), IncN plasmids were broadly distributed among genetically unrelated strains. The horizontal mobility of IncN plasmids has already been reported by Moodley and Guardabassi, who described the spread of a $bla_{CTX\text{-}M\text{-}1}$ IncN plasmid among multiple *E. coli* lineages of porcine and human origin (18). The finding of IncN plasmids that could disseminate by horizontal transmission is worrisome. Our findings concerning the association of ESBL-encoding genes and plasmid replicons are similar to previous findings in humans, chickens, pigs, and dogs (5, 11, 12, 13, 17, 18, 19, 21, 23). The similarity between the ESBL variants we detected and those present in humans in Italy (20) indicates a possible role of poultry in the dissemination of these resistance determinants.

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