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Occurrence of *bla*$_{CTX-M-1}$, *qnr*B1 and virulence genes in avian ESBL-producing *Escherichia coli* isolates from Tunisia

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Avian ESBL-producing *Escherichia coli* isolates have been increasingly reported worldwide. Animal to human dissemination, via food chain or direct contact, of these resistant bacteria has been reported. In Tunisia, little is known about avian ESBL- producing *E. coli* and further studies are needed. Seventeen ESBL-producing *Escherichia coli* isolates from poultry feces from two farms (Farm 1 and farm 2) in the North of Tunisia have been used in this study. Eleven of these isolates (from farm 1) have the same resistance profile to nalidixic acid, sulfonamides, streptomycin, tetracycline, and norfloxacine (intermediately resistant). Out of the six isolates recovered from farm 2, only one was co-resistant to tetracycline. All isolates, except one, harbored *bla*$_{CTX-M-1}$ gene, and one strain co-harbored the *bla*$_{TEM-1}$ gene. The genes *tetA* and *tetB* were carried, respectively, by 11 and 1 amongst the 12 tetracycline-resistant isolates. Sulfonamides resistance was encoded by *sul1*, *sul2,* and *sul3* genes in 3, 17, and 5 isolates, respectively. The *qnrB1* was detected in nine strains, one of which co-harbored *qnrS1* gene. The search for the class 1 and 2 integrons by PCR showed that in farm 1, class 1 and 2 integrons were found in one and ten isolates, respectively. In farm 2, class 1 integron was found in only one isolate, class 2 was not detected. Only one gene cassette arrangement was demonstrated in the variable regions (VR) of the 10 *int2*-positive isolates: *dfrA1- sat2-aadA1*. The size of the VR of the class 1 integron was approximately 250 bp in one *int1*-positive isolate, whereas in the second isolate, no amplification was observed. All isolates of farm 1 belong to the phylogroup A (sub-group A0). However, different types of phylogroups in farm 2 were detected. Each of the phylogroups A1, $B2_2$, $B2_3$ was detected in one strain, while the D2 phylogroup was found in 3 isolates. The virulence genes *iut*A, *fimH*, and *traT* were detected in 3, 7, and 3 isolates, respectively. Two types of gene combination were detected: *iutA*+*fimH*+*traT* in 3 isolates and *iutA*+*fimH* in one isolate. The isolates recovered in farm 1 showed the same profile of PFGE macro-restriction, while isolates of farm 2 presented unrelated PFGE patterns. We conclude that these avian ESBL-producing *E. coli* isolates show homo- and heterogenic genetic background and that plasmids harboring ESBL genes could be involved in the dissemination of this resistance phenotype.

Keywords: ***Escherichia coli*, poultry, *bla*$_{CTX-M-1}$, *qnr*B1, integrons, clonality**

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Introduction

Escherichia coli is a commensal bacterium in humans and animals and considered an indicator of fecal contamination of food. Antimicrobial resistant isolates and resistance genes of *E. coli* can be transferred to humans through the food chain. This transfer represents a potential risk for public health (Alexander et al., 2010; Cortés et al., 2010; Canton et al., 2012; Ryu et al., 2012). The use of broad-spectrum cephalosporins in animals, such as ceftiofur and cefquinome, has been recognized a major driving force for the selection and spread of extended spectrum beta-lactamases (ESBL) (Dutil et al., 2010). In both reservoirs, high prevalence of genes belonging to CTX-M group dominates. Numerous data highlight the extent to which certain ESBL genes, ESBL plasmids or ESBL-producing clones are shared between animals and humans (Naseer and Sundsfjord, 2011).

In recent years, there has been increasing concern in the scientific community about the emergence and dissemination of *E. coli* strains producing ESBLs, especially of the CTX-M class, which are very frequently associated with community infections (Eckert et al., 2004; Livermore et al., 2007; Pitout and Laupland, 2008). Recently, different reports have indicated the dissemination of ESBL-positive *E. coli* strains among the intestinal microbiota of healthy humans (Vinué et al., 2009), in food producing animals, and in food products (Brinas et al., 2005; Blanc et al., 2006; Girlich et al., 2007; Jouini et al., 2007; Li et al., 2007; Smet et al., 2008). These resistant bacteria could be transferred to humans through the food chain. This transfer represents a problem for public health. Comparison of human and animal ESBL-producing isolates is important in enhancing the knowledge of the potential routes of transfer of these bacteria and resistance genes in different ecosystems. In Tunisia, ESBL-producers were initially reported from food samples such as raw chicken meat (Jouini et al., 2007; Ben Slama et al., 2010). More recently, ESBL-producing *E. coli* were described in healthy food animals at farm level (Ben Sallem et al., 2012b, 2013; Mnif et al., 2012). In Tunisia, ESBL-producing bacteria were found in chickens and a dromedary, suggesting that poultry constitutes a major reservoir of ESBL genes. The dominant ESBL gene found was $bla_{\text{CTX-M-1}}$, which was mainly detected with *Inc*I1 replicons (Mnif et al., 2012). A recent study also demonstrated that the $bla_{\text{CTX-M-1}}$ IncI1/ST3 plasmid was dominant in Tunisian chickens and pets (Grami et al., 2013). Finally, recent data showed that 7.3% of Tunisian healthy humans were fecal carriers of CTX-M-1-producing *E. coli*. This finding suggests that foodstuff of poultry origin may contribute to the transmission of the $bla_{\text{CTX-M-1}}$ gene from animals to humans (Ben Sallem et al., 2012b).

The aim of this study was to characterize ESBL producing *E. coli* isolates recovered from feces of healthy chickens in Tunisia by investigating genes encoding ESBL, resistance to tetracycline, sulfonamides and fluoroquinolones as well as content of virulence genes, integrons, and genetic clonality using PFGE.

Materials and Methods

Bacterial Strains

Sixty-five fecal samples were collected from healthy chickens in 2013. Of these samples, 45 came from 58-week-old chickens in a farm located at Sidi Thabet in the North of Tunisia (Farm 1) and 20 from 7-week-old chickens in a farm located at Morneg region (Farm 2). These samples were cultivated on Mac-Conkey agar containing 2 mg/L of cefotaxime and incubated overnight at 37°C. For each sample, one colony with typical *E. coli* trait was picked and re-isolated on Mac-Conkey agar and the phenotypic identification result was confirmed using Api20E (Bio-Mérieux, France).

Antimicrobial Susceptibility Testing and ESBL Identification

Antimicrobial susceptibility testing was carried out using the agar disk diffusion method on Mueller–Hinton agar plates in accordance with the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2012). The following antimicrobial agents were tested (μg/disk): ampicillin (10), amoxicillin (25), amoxicillin-clavulanic acid (20/10), ceftazidime (30), cefotaxime (30), gentamicin (10), kanamycin (30), streptomycin (10), amikacin (30), trimethoprim-sulfamethoxazol (1.25/23.75), tetracycline (30), nalidixic acid (30), ciprofloxacin (5), sulfonamides (200) and chloramphenicol (50). The Double-Disk Synergy Test (DDST) with cefotaxime or ceftazidime in the proximity to amoxicillin-clavulanic acid was used for the screening of ESBL (Clinical and Laboratory Standards Institute, 2012). *E. coli* ATCC25922 was used as ESBL negative and *Klebsiella pneumoniae* 700603 was used as ESBL positive reference strain.

Resistance Genotype

All primers used to characterize the resistance genotype are presented in **Table 1**. The presence of genes encoding TEM, SHV, CTX-M implicated in the beta-lactam resistance was analyzed by PCR (Sáenz, et al., 2004; Batchelor et al., 2005). Amplified DNA fragments were sequenced on both strands and the nucleotide and their deduced amino acid sequences were compared with those included in the Gen-Bank database as well as with those deposited at the website http://www.lahey.org/Studies/ in order to confirm the specific type of β-lactamase gene (Sáenz, et al., 2004; Batchelor et al., 2005). Genes encoding resistance to tetracycline (*tetA*, *tetB*, and *tetC*), sulfonamide (*sul1*, *sul2*, and *sul3*), and quinolones (*qnrA*, *qnrB*, and *qnrS*) were investigated by PCR as reported previously (Sáenz, et al., 2004; Wang et al., 2008; Rocha-Gracia et al., 2010). For positive isolates, PCR products of *qnrB* and *qnrS* were sequenced. The presence and characterization of integrons were studied by PCR of the class 1 and 2 integrase encoding genes as well as the 3′ conserved region (*qacE*Δ1 + *sul1* genes) (**Table 1**) and by PCR and subsequent sequencing of the variables regions (VRs) of these integrons (Sáenz, et al., 2004).

TABLE 1 | Primers of genes encoding resistance genes and integrons used in PCR of resistance genotype.

Primer name	Sequence (5′-3′)	Target gene or region	PCR products (bp)	References
intI1-F	GGGTCAAGGATCTGGATTTCG	*intI1*	483	Sáenz, et al., 2004
intI1-R	ACATGCGTGTAAATCATCGTCG			
Int-F	GGCATCCAAGCAGCAAG	Class 1 integron variable region	Variable	Sáenz, et al., 2004
Int-R	AAGCAGACTTGACCTGA			
intI2-F	CACGGATATGCGACAAAAAGGT	*intI2*	788	Sáenz, et al., 2004
intI2-R	GTAGCAAACGAGTGACGAAATG			
Hep-F	CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron variable region	Variable	Sáenz, et al., 2004
Hep-R	GATGCCATCGCAAGTACGAG			
Qac-F	GGCTGGCTTTTTCTTGTTATCG	*qacE*Δ1-*sul1* region	1125	Sáenz, et al., 2004
SUL1-R	GCGAGGGTTTCCGAGAAGGTG			
SUL1-F	TGGTGACGGTGTTCGGCATTC	*sul1*	789	Sáenz, et al., 2004
SUL1-R	GCGAGGGTTTCCGAGAAGGTG			
SUL2-F	CGGCATCGTCAACATAACC	*sul2*	722	Sáenz, et al., 2004
SUL2-R	GTGTGCGGATGAAGTCAG			
SUL3-F	CATTCTAGAAAACAGTCGTAGTTCG	*sul* 3	990	Sáenz, et al., 2004
SUL3-R	CATCTGCAGCTAACCTAGGGCTTTGGA			
TetA-F	GTAATTCTGAGCACTGTCGC	*tetA*	937	Sáenz, et al., 2004
TetA-R	CTGCCTGGACAACATTGCTT			
TetB-F	CTCAGTATTCCAAGCCTTTG	*tetB*	416	Sáenz, et al., 2004
TetB-R	CTAAGCACTTGTCTCCTGTT			
TetC-F	TCTAACAATGCGCTCATCGT	*tetC*	570	Sáenz, et al., 2004
TetC-R	GGTTGAAGGCTCTCAAGGGC			
aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	*aac(6′)-Ib*	482	Rocha-Gracia et al., 2010
aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT			
QepA-F	GGACATCTACGGCTTCTTCG	*qepA*	671	Rocha-Gracia et al., 2010
QepA-R	CAACTGCTTGAGCCCGTAG			
QnrA-F	GGGTATGGATATTATTGATAAA	*qnrA*	580	Rocha-Gracia et al., 2010
QnrA-R	CTAATCCGGCAGCACTATTA			
QnrBnew-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	468	Wang et al., 2008
QnrBnew-R	ACGATGCCTGGTAGTTGTCC			
QnrS-F	AGTGATCTCACCTTCACCGC	*qnrS*	550	Rocha-Gracia et al., 2010
QnrS-R	CAGGCTGCAATTTTGATACC			
TEM-F	ATTCTTGAAGACGAAAGGGC	*bla*TEM	1150	Sáenz, et al., 2004
TEM-R	ACGCTCAGTGGAACGAAAAC			
SHV-F	CACTCAAGGATGTATTGTG	*bla*SHV	885	Sáenz, et al., 2004
SHV-R	TTAGCGTTGCCAGTGCTCG			
CTXM-Univ-F	CGATGTGCAGTACCAGTAA	*bla*CTXM	585	Batchelor et al., 2005
CTXM-Univ-R	TTAGTGACCAGAATCAGCGG			

Virulence Genotyping

The presence of 30 virulence genes (*fimA, TartT, iutA, MaIX, Ibe, FyuA, BmaE, papGalleleIII, papC, colV, cdtB, papG alleleI, nfaE, SfaS, iha, iss, ire, ehxA, sxt1, sxt2, eltA, fasA, estII, aggC, esat1, cdt, ipah, hly, cnf1*, and *bfp*) was determined by using PCR in all ESBL-positive *E. coli* strains (Chapman et al., 2006; Wu et al., 2007).

Clonal and Phylogenetic Analysis of *E. coli* Isolates

Chromosomal DNA was prepared as previously described using the restriction enzyme *XbaI* (Amersham Life Sciences, Uppsala, Sweden) (Kaufmann, 1998). DNA fragments were separated by electrophoresis in 1.2% agarose gels (pulsed-field agarose certified; Bio-Rad, Hemel Hempstead, United Kingdom) and 0.5 X Tris-borate-EDTA buffer using a contour-clamped homogeneous electric field (CHEF-DRIII system; Bio-Rad) under the following electrophoresis conditions: 12°C at 6 V/cm for 27 h with pulse times ranging from 10 to 40 s. Clonal relationships were established following Tenover criteria (Tenover et al., 1995). *E. coli* Isolates were allotted to phylogenetic groups A, B1, B2, or D, using a triplex PCR assay targeting the *chuA*, *yjaA* genes and the DNA fragment TSPE4.C2 (Clermont et al., 2000). Strains were sub-grouped according to Escobar-Paramo et al. (2006): subgroup A0: *chuA*-, *yjaA*-, and TspE4.C2-; subgroup A1: *chuA*-, *yjaA*+, and TspE4.C2-;

group B1: *chuA*-, *yjaA*+/-, and TspE4.C2+; subgroup $B2_2$: *chuA*+, *yjaA*+, and TspE4.C2-; subgroup $B2_3$: *chuA*+, *yjaA*+, and TspE4.C2+; subgroup D1: *chuA*+, *yjaA*-, and TspE4.C2-; subgroup D2: *chuA*+, *yjaA*, and TspE4.C2+. Appropriate positive and negative controls were included in the assay.

Results

Occurrence of ESBL-producing *E. coli* Isolates and Antibiotic Susceptibility

Eleven (24%) and 6 (30%) ESBL-producing *E. coli* isolates were detected in the 45 and 20 fecal samples collected in farm 1 and in farm 2, respectively. In addition to ESBL production, isolates in farm 1 have the same profile of resistance to nalidixic acid, norfloxacine (intermediate), trimethoprim-sulfamethoxazole, sulfonamides, streptomycin and tetracycline. But, they remained susceptible to imipenem, gentamicin, tobramycin and chloramphenicol. With the exception of one strain that was co-resistant to tetracycline, isolates, from farm 2, were susceptible to all non beta-lactam antibiotics (**Table 2**).

Gene Coding for the Production of ESBL

PCR and sequencing showed that all strains, except one, harbored the $bla_{\text{CTX-M-1}}$ genes. One strain co-harbored $bla_{\text{TEM-1}}$ gene. The bla_{SHV} gene was not detected.

Occurrence of Class 1 and 2 Integrons

In farm 1, class 1 and 2 integrons were found in one and ten isolates, respectively. In farm 2, class 1 integron was found in only one isolate, while class 2 was not detected. Amplification of the VRs of class 2 integrons showed identical DNA fragments with an approximate size of 2000 bp. All these VRs contained a unique gene cassette arrangement, being *dfrA1- sat2-aadA1*, encoding resistance for trimethoprim, streptothricin and streptomycin, respectively. In one isolate, the VR of class 1 integron was amplified and yielded a DNA fragment of ca. 250 bp, whereas no amplification was observed in the second *int1*-positive isolate.

Determination of Phylogroups and Virulence Factors

In farm 1, all isolates were found to belong to phylogroup A (subgroup A0); however, different phylogroups were detected in farm 2 (A1, $B2_2$, and $B2_3$, each in one isolate, and D_2, in 3 isolates). The virulence genes *iut(A), fimH*, and *traT* were detected in 3, 7, and 3 isolates, respectively. The other genes were not detected in our collection. Two types of gene combination were detected: *iutA+fimH+traT* (in 3 isolates); *iutA+fimH* (in one isolate).

Genes Encoding Tetracycline-, Sulphonamide, and Fluroquinolones Resistance

Amongst the 12 tetracycline-resistant isolates, eleven and one carried *tetA* and *tetB*, respectively. Sulfonamides resistance was encoded by *sul1*, *sul2* and *sul3* genes in 3, 17 and 5 isolates, respectively. Gene *qnrB1* was detected in 9 isolates, one of them co-harbored *qnrS1* gene.

PFGE Typing

All isolates from farm 1 were clonally related, while isolates of the farm 2 were genetically unrelated (**Figure 1**).

TABLE 2 | Phenotypic and molecular characteristics of the 17 ESBL-producing *E. coli* isolates.

E. coli strain	Resistance profil*	Phylogenetic group	β-Lactamase (s)	Int**	VR** (bp)	*tet*	*sul*	PMQR**	Virulence factors	PFGE type
Ec139	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec149	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 1-2*	-	*iut A*	A
Ec174	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	-	*iut A*	A
Ec143	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec147	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M1	2	2000	A	*sul 2*	-	*iut A*	A
Ec154	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec146	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M1	2	2000	A	*sul 1-2*	-	*iut A*	A
Ec172	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1+ TEM-1	1	250	A	*sul 2-3*	*qnrB1*	*iut A, traT, fimH*	A
Ec156	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1, qnrS1*	-	A
Ec151	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec173	NA, Nor^I, Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec67	-	A1	CTX-M-1	1	-	-	*sul 1-2*	-	*fimH*	B
Ec68	TET	$B2_2$	-	-	-	B	*sul 2-3*	-	*iut A, traT, fimH*	C
Ec69	-	D_2	CTX-M-1	-	-	-	*sul 2*	*qnrB1*	*iut A, traT, fimH*	D
Ec71	-	$B2_3$	CTX-M-1	-	-	-	*sul 2-3*	*qnrB1*	*iut A, fimH*	E
Ec 72	-	D_2	CTX-M-1	-	-	-	*sul 2-3*	-	*fimH*	F
Ec 76	-	D_2	CTX-M-1	-	-	-	*sul 2-3*	-	*fimH*	G

*Resistance to other antibiotic in addition to ESBL production, NA, nalidixic acid; Nor, Norfloxacine; Sxt, trimethoprim-sulfamethozole; S, streptomycin; Tet, tetracycline.
**Int, integron class; VR, variable region; PMQR, Plasmid Mediated Quinolone Resistance.

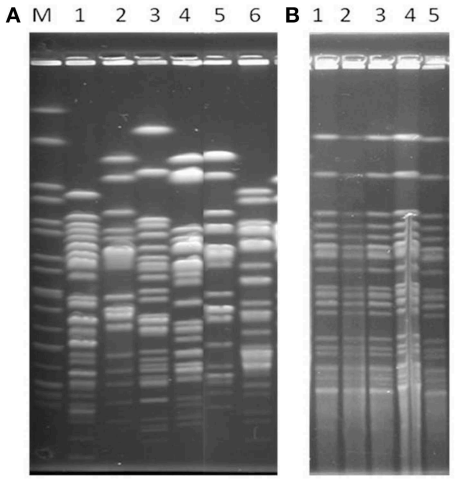

FIGURE 1 | *Xba*I-PFGE profiles of representative *E. coli* isolates. (**A**, Unrelated isolates) Lanes M, *Xba*I-digested DNA of *Salmonella enterica* serovar Braenderup H9812, used as size standard; Lanes 1–6, Ec 67, Ec68, Ec69, Ec71, Ec72; (**B**, Clonal isolates) Lanes 1 to5, EC139, EC149, Ec 174, Ec143, Ec147, respectively.

Discussion

We collected 65 samples of feces from 45 reproductive 58-weeks-old chickens in a public farm in the region of Sidi Thabet (30 Km Nord-West of Tunis, Tunisia) (Farm1) and 20 samples of 7-weeks-old breeder chickens in the region of Morneg (Farm 2). Seventeen samples (26.1%) contained cefotaxime-resistant isolates, 11 (24%) and 6 (30%) positives samples were observed in farms 1 and 2, respectively. The frequency of cefotaxime-resistant isolates is similar to those reported by other authors worldwide (2%; 31.7%) (Dierikx et al., 2013; Randall et al., 2014), but lower than those found by other studies in Tunisia (42% and 45, 5%) (Ben Sallem et al., 2012b; Mnif et al., 2012). In our study, all isolates from farm 1 exhibited the same resistance profile to nalidixic acid, trimethoprim-sulfamethoxazole, sulfonamides, streptomycin, tetracycline and norfloxacine (intermediately resistant). These resistance markers are often reported in previous Tunisian studies (Ben Slama et al., 2010; Mnif et al., 2012). All isolates were susceptible to imipenem, gentamicin, kanamycin and chloramphenicol. Indeed, low rates of resistance to these antibiotics have been reported worldwide (Grami et al., 2013). However, the 6 isolates from farm 2, except one that was co-resistant to tetracycline, were susceptible to all antibiotics tested. Worldwide, the CTX-M group has been emerged as the predominant determinant encoding ESBL production in human and animal *Enterobacteriaceae*, especially *E. coli* (Girlich et al., 2007; Coque et al., 2008; Nicolas-Chanoine et al., 2008; Dahmen et al., 2012, 2013). The $bla_{\text{CTX-M-1}}$ gene was amplified in 16 out of 17 isolates; in addition, one isolate co-harbored the $bla_{\text{TEM-1}}$ gene. This result is in agreement with those of other Tunisian findings (Jouini et al., 2007, 2009; Ben Slama et al., 2010; Ben Sallem et al., 2012b, 2013; Mnif et al., 2012). The high rate occurrence of *E. coli* harboring $bla_{\text{CTX-M-1}}$ in poultry and other food products of animal origin can contribute to the transmission of this gene or these strains to humans. Indeed, it was recently demonstrated that *E. coli* containing CTX-M-1 was identified in 7.3% of healthy Tunisians (Ben Sallem et al., 2012a). Identical or closely related isolates from humans and animals have been previously reported in Netherlands, suggesting a likely transmission of ESBL-*E. coli* isolates from poultry to human, most probably via the food chain (Overdevest et al., 2011). More recently, Chinese data showed a high occurrence of CTX-M-14 in isolates from animals where this enzyme is very widespread in human isolates (Zheng et al., 2012). In the EC68 strain, no *bla* gene was detected. This strain could harbor a gene not investigated in our study. Further investigation must be undertaken. Antibiotic resistance, especially the multiresistance, has been mainly linked to the dissemination of linked genes encoding resistance inserted in mobile genetic elements, mainly integrons. Integron of class 1 was found in two isolates while integron of class 2 in ten isolates. Our results are not in agreement with other findings, which showed the dominance of integron of class 1 in animal-derived *E. coli* or in animal products as well as human isolates (Machado et al., 2005; Soufi et al., 2009, 2011; Ben Slama et al., 2010; Cergole-Novella et al., 2010). Two hypotheses could explain our finding: firstly, the real absence of class 1 integrons in the integron-free isolates, as reported by other authors (Jouini et al., 2007; Ben Sallem et al., 2012b). Secondly, the presence of insertion sequences, such as *IS*26, truncating the *intI* and thus, leading to inhibition of PCR amplification. In the case of truncated *intI* gene by *IS*26, the use of other primers was proposed (Marchant et al., 2013). Owing to a number of financial limitations, we could not realize these PCRs actually. The then *int2*-positive isolates presented an identical gene cassette array in their VRs: *dfrA-sat2-aadA1*, being frequent in other studies (Soufi et al., 2009, 2011). The integrase Int2 is not functional and thus unable to integrate new gene cassettes into the variable region, other than those already present (Partridge et al., 2009). The variable region of the class 2 integron mainly carries *dfrA1* (encoding trimethoprim resistance), *sat1* (encoding streptothricin resistance) and *aadA1* (encoding streptomycin/spectinomycin resistance) (Partridge et al., 2009; Soufi et al., 2009, 2011). The size of the VR of class 1 integron in EC172 was approximately 250 bp, a fragment that cannot correspond to any gene cassette of the known ones (Ravi et al., 2014). The VR of class 1 integron in EC67 was not amplified; this might suggest an empty VR or mismatches of used primers.

Tetracycline resistance was encoded by *tetA* and *tetB* genes in 11 and one tetracycline-resistant isolates, respectively. This finding was also reported by other studies (Koo and Woo, 2011; Xibiao et al., 2011), while the *tetC* gene is rarely reported (Skoèková et al., 2013). The *sul1*, *sul2* and *sul3* genes were detected in 3, 17, and 5 isolates, respectively, not only amongst sulfonamid resistant isolates, but also in the six-sulfonamide susceptible isolates. In other reports, *sul1* is most frequently reported followed by *sul2* gene while *sul3* gene is generally less so (Sköld, 2000; Perreten and Boerlin, 2003; Hammerum et al., 2006; Trobos et al., 2008). Resistance toward quinolone and fluoroquinolones is mainly due to target mutations in quinolone

resistance determining region (QRDR) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerases IV (*parC* and *parE*) (Hawkey, 2003). However, Plasmid Mediate Quinolone Resistance (PMQR) genes such as *qnrA, qnrB, qnrC, qnrD, qnrS, qepA,* and *aac(6′)Ib-cr* have been increasingly reported in bacterial pathogens since 2000 (Strahilevitz et al., 2009). In our study, among 11 isolates resistant to nalidixic acid and intermediately susceptible sensibility to norfloxacine, seven isolates carried *qnrB1* gene and one also co-heberged *qnrS1* gene. These two genes were often reported by other authors worldwide (Ben Sallem et al., 2013, 2014; Ferjani et al., 2014) in *E. coli* producers of CTX-M from human and animal origin. In our study, we did not consider *qepA* and *aac (6′)-Ib-cr* genes, while other authors reported their presence in *E. coli* from animal origin (Ma et al., 2009; Xie et al., 2014). The *qnr* gene types have no big effect on the increase of minimal inhibitory concentration (MIC) of fluoroquinolones (ciprofloxacine and norfloxacine) and were generally not detected by disk diffusion method. Indeed, the resistance toward quinolones in 7 amongst the 11 nalidixic acid resistant isolates could be explained by these *qnr* genes but it could also certainly explained by the presence of chromosomal mutations in QRDR of GyrA and/or ParC (Abbassi et al., 2010). The quinolone-susceptible isolate containing *qnrB1* gene confirmed the low level expression of this resistance (in term of increase of MIC).

Concerning the distribution of pathogenic *E. coli* strains according to phylogroups, it is well-known that pathogenic strains producing extra-intestinal infections (ExPEC) belong mainly to the B2 group and less to the D group. They are responsible for meningitidis, abscess, peritonitis, septicemia and urinary tract infections (Le Gall et al., 2007; Nandanwar et al., 2014); while, groups A and B1 *E. coli* strains are considered non-virulent commensal strains (Ewers et al., 2007). All the strains of farm 1 were found to belong to the phylogroup A (sub-type A0), while, the strains of farm 2 to phylogroups A (A1, 1 isolate), D (D2, 3 isolates) and B2 ($B2_2$, 1 isolate; $B2_3$, 1 isolate). In the literature, the majority of animal *E. coli* isolates producing ESBL belonged to phylogroup A and B1 (Mnif et al., 2012; Huber et al., 2013) contrary to human isolates, which mainly belonged to B2 phylogroup. The low number of virulence genes detected was in relation with the appurtenance of our isolates (12/17) to the phylogroup A. Strains of phylogroups B2 and D were somewhat distinguished from strains of phylogroup A by the occurrence of the fimbrial *fimH* gene and the serum resistance-associated outer membrane (*traT*). The low number of genes detected could be explained by fitness notion or the competitively of strains. Indeed, these avian intestinal *E. coli* isolates were under selective presser by antibiotics. This selective pressure enhances the expansion and maintenance of antibiotic-resistant strains rather than virulent ones.

PFGE showed that the 11 isolates of the farm 1 were indistinguishable, while isolates of farm 2 were unrelated. The cloanlity of these isolates would evoke a strong power of dissemination of clonal isolates in this poultry breeding. The difference in genetic contents in isolates of farm2 (*sul* genes, *qnr* genes and virulence genes) might be due to independent acquisition of genes carried by plasmids or integrons.

Conclusion

Despite the limited samples analyzed in this study, that might not reflect the real epidemiological situation of avian ESBL-producing *E. coli* in these two farms, our study showed two typical epidemiologic characteristics of ESBL-producing bacteria. Firstly, clonal isolates are disseminated within the same farm. Secondly, "singleton" isolates occur with limited ability of spread, evoking the potential horizontal transfer of ESBL genes between different *E. coli* populations. Plasmids or integrons might be implicated in the mobilization of $bla_{\text{CTX-M-1}}$ genes among avian isolates. Further studies should be performed in the future to track the evolution of ESBL types and their frequencies in different ecosystems.

Author Contributions

HK, MA, RM, SH, NC, and IB: Conceiving and designing the study, collecting and interpreting the data, and writing the article. SS, RS, IJ, and SF: interpreting the data, revising the article.

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