Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

Article

Genomic characterization of ESBL-producing *Escherichia coli* isolates belonging to a hybrid aEPEC/ExPEC pathotype O153:H10-A-ST10 *eae*-beta1 occurred in human diarrheagenic isolates, meat, poultry and wildlife

Dafne Díaz-Jiménez [1+], Isidro García-Meniño [1+], Alexandra Herrera [1], Vanesa García [1,4], Ana María López-Beceiro [2], María Pilar Alonso [3], Jorge Blanco [1], and Azucena Mora [1,*]

[1] Laboratorio de Referencia de *Escherichia coli* (LREC), Departamento de Microbioloxía e Parasitoloxía, Facultade de Veterinaria, Universidade de Santiago de Compostela (USC), Lugo, Spain; dafne.diaz@usc.es; isidro.garcia@usc.es; alex.herrera.est@gmail.com; vanesag.menendez@usc.es; jorge.blanco@usc.es; azucena.mora@usc.es

[2] Departamento de Anatomía, Produción Animal e Ciencias Clínicas Veterinarias, Facultade de Veterinaria, Universidade de Santiago de Compostela (USC), Lugo, Spain; anam.lopez.beceiro@usc.es

[3] Servicio de Microbiología, Hospital Universitario Lucus Augusti (HULA); Lugo, Spain; Pilar.Alonso.Garcia@sergas.es

[4] Present address: Department of Veterinary and Animal Sciences, Section of Veterinary Clinical Microbiology, University of Copenhagen, København, Denmark

+ These authors have contributed equally to this work.

* Correspondence: azucena.mora@usc.es; Tel.: (+34 982822110)

Abstract: Different surveillance studies (2005-2015) on the presence of ESBL-producing *E. coli* in the northwest Spain revealed that *eae*-positive isolates of serotype O153:H10 were periodically detected in meat (of beef, chicken and pork), and also implicated in human diarrhea. This study aimed: i) to characterize the degree of relatedness between human and animal isolates; ii) to know if this was a geographically restricted or disseminated genetic lineage. Thirty-two isolates were conventionally typified as O153:H10-A-ST10 *fim*H54, *fimAv*$_{MT78}$, *traT* and *eae*-beta1, being 21 of those CTX-M-32 or SHV-12 producers. PFGE comparison of their macrorestriction profiles showed high similarity (>85%). The plasmidome analysis revealed a stable combination of IncF (F2:A-:B-), IncI1 (STunknown) and IncX1 plasmid types, together with non-conjugative Col-like. Besides, the core genome investigation based on the cgMLST scheme from Enterobase, proved close relatedness between isolates of human and animal origin. Our results demonstrate that a hybrid MDR aEPEC/ExPEC of clonal group O153:H10-A-ST10 (CH11-54) would be playing a successful role in spreading ESBLs (CTX-M-32) in our region within different hosts, including wildlife. It would be potentially implicated in human diarrhea via food (meat) transmission. Importantly, we proved genomic evidence of a related hybrid aEPEC/ExPEC in other countries.

Keywords: *Escherichia coli*, ESBL, hybrid pathotype, ExPEC, EPEC, MDR, ST10, O153, Enterobase

© 2020 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

1. Introduction

Escherichia coli is a normal inhabitant of the human and animal intestinal tract. However, *E. coli* can also act as a pathogen in a broad range of conditions, from diarrheagenic diseases to extraintestinal infections. Unlike extraintestinal pathogenic *E. coli* (ExPEC), with no specific virulence determinants for each subtype, the diarrheagenic *E. coli* (DEC) categories are characteristically defined by certain pathotype-specific virulence markers [1,2]. Thus, enteropathogenic *E. coli* (EPEC) are carriers of the *eae* gene, as part of the pathogenicity island LEE, codifying a protein called intimin. The intimin is responsible for the intimate adherence of the bacteria to the enterocyte membranes and, eventually, for the attaching and effacing (AE) lesion of the brush-border microvilli [3]. The variable C-terminal-encoding sequence of *eae* defines more than 30 distinct intimin types and subtypes associated with tissue tropism [4,5]. EPEC are further classified as typical (tEPEC), when they carry an EPEC adherence factor (EAF) plasmid that encodes adherence mediated by the bundle forming pilus (BFP), while atypical EPEC (aEPEC) produce the AE lesion but do not express BFP [4,6]. Currently, aEPEC isolates are emerging enteropathogens detected worldwide and isolated from different niches (animal species, environment, and food samples), while the main reservoir of tEPEC isolates are humans [7,8].

Antimicrobial resistance is a serious global concern which involves the health care system, food production and environmental integrity [9]. In fact, it is assumed that antimicrobial drug use in the livestock sector plays an important role in the spread of extended-spectrum beta lactamases (ESBL)-producing *E. coli* thought the food chain to humans [10,11]. The genomic plasticity of *E. coli* is the consequence of the important role played by mobile genetic elements (MGEs) such as plasmids, bacteriophages, pathogenicity islands, transposons and insertion sequence elements in the evolution of the bacteria [12]. As a result, hybrid *E. coli* pathotypes unpredictably emerge, given the mobility of most of the genes encoding virulence and antimicrobial resistance (AMR) [12,13]. Since 2011, when a novel Shiga-toxin-producing *E. coli* (STEC) belonging to serotype O104:H4, with virulence features (VF) common to the enteroaggregative *E. coli* (EAggEC), and CTX-M-15 producer was identified as the one involved in the large German outbreak [14], the concept of pathotype has been questioned. Currently, classical and new approaches, such as whole genome sequencing (WGS), are being used to enhance the understanding the evolution of this highly adaptable species [13,15].

From different in-house surveillance studies on the presence of ESBL-producing *E. coli* in the northwest of Spain (2005-2015), we noticed that *eae*-positive isolates of the serotype O153:H10 were periodically recovered from meat, wildlife, and avian farm environment. We also found them involved in human diarrhea. This study aimed: i) to characterize the degree of relatedness between human and animal isolates; ii) to know if this was a geographically restricted or disseminated genetic lineage.

2. Results

Thirty-two *eae*-positive *E. coli* (21 ESBL and 11 non-ESBL) belonging to the serotype O153:H10 constituted the collection of study. They were detected within different surveys in the period 2005 to 2015: 14 from human stools, eight from beef meat, seven from chicken meat, and one each of pork meat, wildlife (fox feces) and poultry farm environment (Table S1).

2.1. Conventional typing

Table 1 summarizes the main traits determined by conventional typing for the 32 isolates. All were positive for the intimin *eae*-beta1, but negative for *bfpA* gene, conforming the aEPEC pathotype. Other virulence genes defining verotoxigenic (VTEC), enteroinvasive (EIEC), enteroaggregative (EAggEC) or enterotoxigenic (ETEC) pathotypes were not detected; however, the $fimAv_{MT78}$ gene, which is a virulence *locus* that codify a *fimA* variant MT78 of type 1 fimbriae [16] was present in all isolates. Besides, the *traT* gene that codifies an outer membrane protein implicated in serum survival [17] was also present in 17 of the isolates (Table 1). By means of the serotype, phylogroup, ST and clonotyping, the isolates were assigned to the clonal group O153:H10-A-ST10 (CH11-54).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

The highest rates of AMR were to: ampicillin (75%; 24/32), cefuroxime (68.7%; 22/32), cefotaxime (65.6%, 21/32), ceftazidime (65.6%, 21/32), cefepime (59.4%, 19/32) and gentamicin (59.4%, 19/32). The ESBL-typing determined that 19 isolates were CTX-M-32 and two SHV-12 (Table 1).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

1

Table 1. Phenotypic and genotypic characterization of 32 aEPEC O153:H10-A-ST10 (CH11-54) isolates

Sample origin	Code[a]	Year	Geographic origin	Virulence gene profile	Resistance profile	bla_{ESBL} type
Pork meat	*LREC-122	2011	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	*LREC-115	2009	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Chicken meat	FV 19517	2009	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	*LREC-118	2009	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	*LREC-110	2010	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	FV 14703	2010	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB, FOF	CTX-M-32
Chicken meat	LREC-126	2010	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Chicken meat	*LREC-123	2010	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Beef meat	*LREC-119	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Beef meat	*LREC-117	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Beef meat	4-3a	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ	SHV-12
Beef meat	85-5a	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, GEN	-
Beef meat	*LREC-125	2008	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, FEP	CTX-M-32
Beef meat	*LREC-114	2008	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Beef meat	65-6a	2009	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Beef meat	*LREC-120	2011	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP	SHV-12
Wildlife (Fox)	*LREC-111	2015	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Poultry farm	*LREC-127	2010	Pontevedra	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Human	*LREC-116	2006	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	*LREC-113	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	*LREC-121	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	*LREC-124	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	31952. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Human	32651. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	NAL, CIP	-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

Human	32884. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CAZ, AMC, SXT	-
Human	34535. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	NAL, CIP	-
Human	39044. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Human	21011. 08	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Human	38506. 08	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	CIP	-
Human	40237. 08	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	NAL, CIP	-
Human	*LREC-112	2011	Santiago de Compostela	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, NAL	CTX-M-32
Human	55515.12	2012	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, GEN	-

2 [a] Strains further analyzed by WGS are those marked with (*); [b] ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP),
3 cefoxitin (FOX), gentamicin (GEN), tobramycin (TOB), fosfomycin (FOF), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP) and nalidixic acid (NAL)
4

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

The PFGE comparison of the *XbaI*-macrorrestriction profiles of the ESBL-producing aEPEC isolates revealed high similarity. Thus, all but one clustered with an identity >85% in the dendrogram shown in Figure 1. It is of note that three human clinical isolates, recovered in different years, clustered each with a fox (95.2% of similarity) and with two beef meat isolates (100% and 97.6% of similarity, respectively).

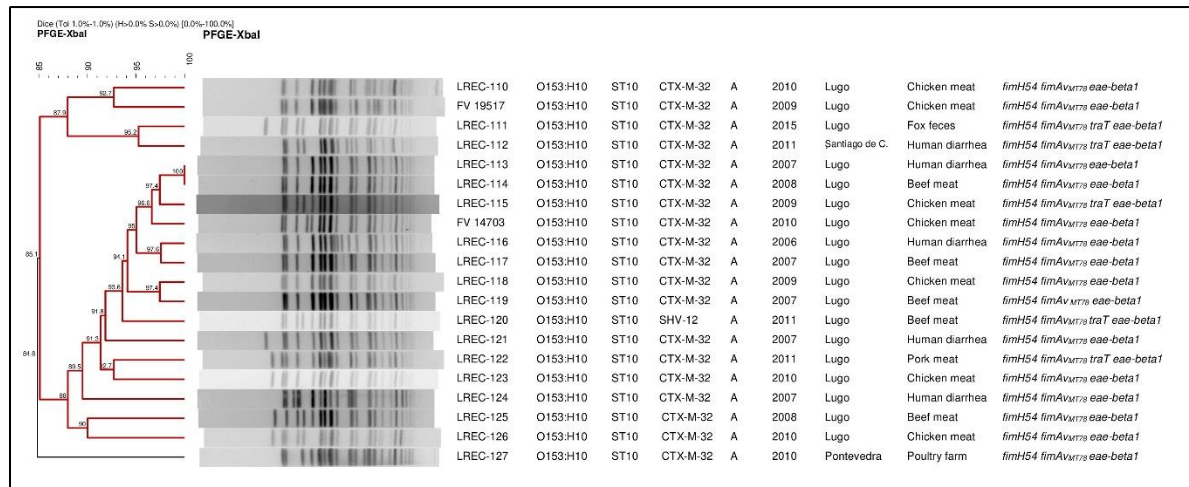

Figure 1. PFGE of *XbaI*-digested DNA from 20 ESBL-producing aEPEC isolates of the clonal group O153:H10-A-ST10 (one autodigested). On the right of the dendrogram: Isolate designation, O:H serotype, ST, ESBL type, phylogroup, year of isolation, geographic origin, source and virulence-gene profile

2.2 Whole genome sequencing (WGS)

To further investigate the virulence profile, resistome, plasmid content and relatedness, 17 representative aEPEC/ExPEC isolates of different origins were WG sequenced. The *de novo* assembled contigs were then typed *in silico* using the Enterobase tools (Table S2), as well as the Center for Genomic Epidemiology (CGE) databases (Table 2).

SerotypeFinder and Enterobase predictions corroborated O and H antigens, with the exception of LREC-120 and LREC-121, for which O153 was solved by serotyping. MLST (CGE and Enterobase), CHtyper and ClermonTyping also confirmed conventional data for ST (10), CH (11-54) and phylogroup (A) (Table 2, Table S2). Additionally, the wgST, cgST, and rST of the genomes were determined using the schemes of Enterobase based on 25,002; 2,513 and 53 loci, respectively (Table S2). WgMLST and cgMLST are powerful schemes with extreme and high resolution, respectively, which determined different STs for each of the 17 genomes analyzed, while rST (medium resolution) established the same ST (2021) for all genome but for LREC-127 (58738) (Table S2).

VirulenceFinder corroborated the hybrid pathotype nature of the isolates, predicting in all genomes the *eae* gene (intimin) together with other components encoded in the LEE pathogenity island, as well as the increased serum survival gene *iss* recognized for its role in ExPEC virulence [18]. Besides, the *astA* gene, which encodes the heat-stable enterotoxin 1, was also present in all 17 isolates (Table 2).

ResFinder identified the genes associated to resistances observed *in vitro* (acquired resistances for beta-lactams, aminoglycosides, and point mutations for quinolones). Only, the $bla_{CTX-M-32}$ was not predicted *in silico* for LREC-112 and LREC-119, but by conventional sequencing. Furthermore, ResFinder determined other acquired resistances which had not been tested *in vitro*, such as to phenicols and macrolides in all genomes, and to tetracyclines in 16 out of the 17 genomes (Table 2).

Based on replicon identification, PlasmidFinder revealed a homogenous profile of four/five plasmid types. Thus, the concomitant presence of IncF (F2:A-:B-), IncI1 (STunknown) and IncX1, together with non-conjugative Col156-like plasmids, was detected in 15 of 17 genomes. Four of those 15 genomes were also carriers of Col (MG828)-like plasmids (Table 2).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

In the asymmetric distance matrix on the cgMLST scheme from Enterobase, based on the presence/absence of 2,513 genes, the 17 genomes showed <20 differences (range 5-19) in relation to the human diarrheagenic isolate LREC-113 (Table 3, Figure 2). We also looked into the static Hierchical Clustering (HierCC) designations in Enterobase. The 17 genomes were assigned into the same HierCC HC50 (37600), which means all strains in this cluster have links no more than 50 alleles apart. Besides, using HC20, three human genomes (LREC-113, LREC-116, LREC-124) and two beef meat (LREC-119, LREC-125) clustered together (37606) with links no more than 20 alleles apart (Table S3). A dendrogram based on the SNPs of the core genomic regions present in 90% of the compared genomes and using LREC-113 as reference, was also built in Enterobase, downloaded and modified with FigTree v1.4.3 (Figure 3). Within 1,068 variant sites, the number of SNPs was <62 for 13 of the 17 genomes (Table S3).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

Table 2. *In silico* characterization of 17 *E. coli* genomes from the study collection using CGE databases and ClermonTyping

Code	Serotype[1]	Phylo group[2]	CHType[3]	ST[4]	Plasmid content Inc group (pMLST)[5]	Acquired resistances (black) and point mutations (blue)[6]	Virulence genes[7]
LREC-110	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-111	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156 Col (MG828)	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-112	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncX1 Col156 Col (MG828)	*bla*$_{CTX-M-32;}$ *aadA1; catA1; mdf(A); tet(A); gyrA* S83L	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP*
LREC-113	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, tir*
LREC-114	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156 Col (MG828)	*bla*$_{CTX-M-32,}$ *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tir*
LREC-115	O153:H10	A	11-54	10	IncF (F2:A-:B-)	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

					IncI1 (STunknown) IncX1 Col156		
LREC-116	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, tccP, tir*
LREC-117	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156	*bla*$_{CTX-M-32}$; *aadA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, tccP, tir*
LREC-118	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156 Col(MG828)	*bla*$_{CTX-M-32,}$ *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-119	O153:H10	A	11-54	10	Col156	*bla*$_{CTX-M-32,}$ *aadA1; catA1; mdf(A)*	*astA, eae, espA, espB, gad, iss, nleA, tccP, tir*
LREC-120	O153:H10	A	11-54	10	IncI1 (ST22-CC2) IncQ1 IncX1 Col156 Col (MG828)	*bla*$_{SHV-12}$; *aadA1, aadA2; catA1, cmlA1; mdf(A); sul3; tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-121	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

					Col156		
LREC-122	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156 Col (MG828)	*bla*$_{CTX\text{-}M\text{-}32}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-123	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156 Col (MG828)	*bla*$_{CTX\text{-}M\text{-}32}$, *bla*$_{TEM\text{-}1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-124	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 IncY Col156	*bla*$_{CTX\text{-}M\text{-}32}$, *bla*$_{TEM\text{-}1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, tccP, tir*
LREC-125	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156	*bla*$_{CTX\text{-}M\text{-}32}$; *aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-127	O153:H10	A	11-54	10	IncF (F2:A-:B-) IncI1 (STunknown) IncX1 Col156 Col (MG828)	*bla*$_{CTX\text{-}M\text{-}32}$, *bla*$_{TEM\text{-}1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

[1]Serotypes, [3]clonotypes, [4]sequence types, [5]replicon/plasmid STs, [6]acquired antimicrobial resistance genes and/or chromosomal mutations, [7]virulence genes were determined using SerotypeFinder 2.0, CHtyper 1.0, MLST 2.0, PlasmidFinder 2.0, pMLST 2.0, ResFinder 3.1 and VirulenceFinder 2.0 online tools at the CGE, respectively. While [2]phylogroups were predicted using the ClermonTyping tool at the Iame-research Center web.

[1]Serotypes: underlined and in red those (LREC-121, LREC-120) that were not predicted (ONT) by SerotypeFinder but assigned as O153 by conventional typing.

[6]Resistome: Acquired resistance genes: beta-lactam: bla_{TEM-1A}, $bla_{CTX-M-32}$, bla_{SHV-12}; aminoglycosides: *aac(3)-IIa, aadA1, aadA2;* phenicols: *catA1, cmlA1;* macrolides: *mdf(A)*; sulphonamides: *sul3*; tetracycline: *tet(A).* Point mutations (marked in blue): quinolones and fluoroquinolones: *gyrA* S83L: TCG-TTG. Underlined and in red those $bla_{CTX-M-32}$ genes (LREC-112, LREC-119) that were not predicted by ResFinder but determined in conventional typing

[8]Virulence genes: *astA*: EAST-1, *eae*: intimin, *espA*: type III secretions system, *espB*: secreted protein B, *espF*: type III secretions system, *gad*: glutamate descarboxylase, *iss*: increased serum survival, *mchF*: ABC transporter protein MchF, *nleA*: non LEE encoded effector A, *tccP*: Tir cytoskeleton coupling protein, *tir*: translocated intimin receptor protein. bp: base pairs; CHType: clonotype (*fumC-fimH*); ST: sequence type according to Achtman scheme; pMLST: plasmid sequence type.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

Table 3. Asymmetric distance matrix based on the cgMLST scheme from Enterobase in which D (a, b) equals all sites that are present in (b) and different from (a)

		LREC-110	**LREC-111**	**LREC-127**	**LREC-112**	**LREC-113**	**LREC-120**	**LREC-117**	**LREC-121**	**LREC-119**	**LREC-116**	**LREC-115**	**LREC-114**	**LREC-123**	**LREC-122**	**LREC-124**	**LREC-125**	**LREC-118**
Genome code / cgMLST		37600	37601	37602	37605	37606	37607	37609	37610	37611	37612	37613	37614	37615	37616	37617	37618	38299
LREC-110	37600	0	14	17	19	13	12	18	19	12	15	27	8	15	15	18	16	14
LREC-111	37601	14	0	21	23	16	20	22	23	16	19	30	11	19	18	22	20	17
LREC-127	37602	17	21	0	24	9	22	14	16	9	11	24	15	13	13	15	13	13
LREC-112	37605	19	23	24	0	19	25	24	25	18	21	33	17	22	22	24	23	21
LREC-113	37606	13	16	9	19	0	18	9	11	5	6	18	10	9	8	11	9	8
LREC-120	37607	12	20	22	25	18	0	23	23	18	20	33	14	20	20	24	22	19
LREC-117	37609	18	22	14	24	9	23	0	17	8	12	22	15	14	13	14	12	13
LREC-121	37610	19	23	16	25	11	23	17	0	10	13	25	15	16	16	16	14	14
LREC-119	37611	12	16	9	18	5	18	8	10	0	6	17	10	9	9	7	6	8
LREC-116	37612	15	19	11	21	6	20	12	13	6	0	22	12	11	11	13	11	10
LREC-115	37613	27	30	24	33	18	33	22	25	17	22	0	22	24	23	23	15	20
LREC-114	37614	8	11	15	17	10	14	15	15	10	12	22	0	13	12	15	14	11
LREC-123	37615	15	19	13	22	9	20	14	16	9	11	24	13	0	7	15	13	8
LREC-122	37616	15	18	13	22	8	20	13	16	9	11	23	12	7	0	15	13	9
LREC-124	37617	18	22	15	24	11	24	14	16	7	13	23	15	15	15	0	12	14
LREC-125	37618	16	20	13	23	9	22	12	14	6	11	15	14	13	13	12	0	12
LREC-118	38299	14	17	13	21	8	19	13	14	8	10	20	11	8	9	14	12	0

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

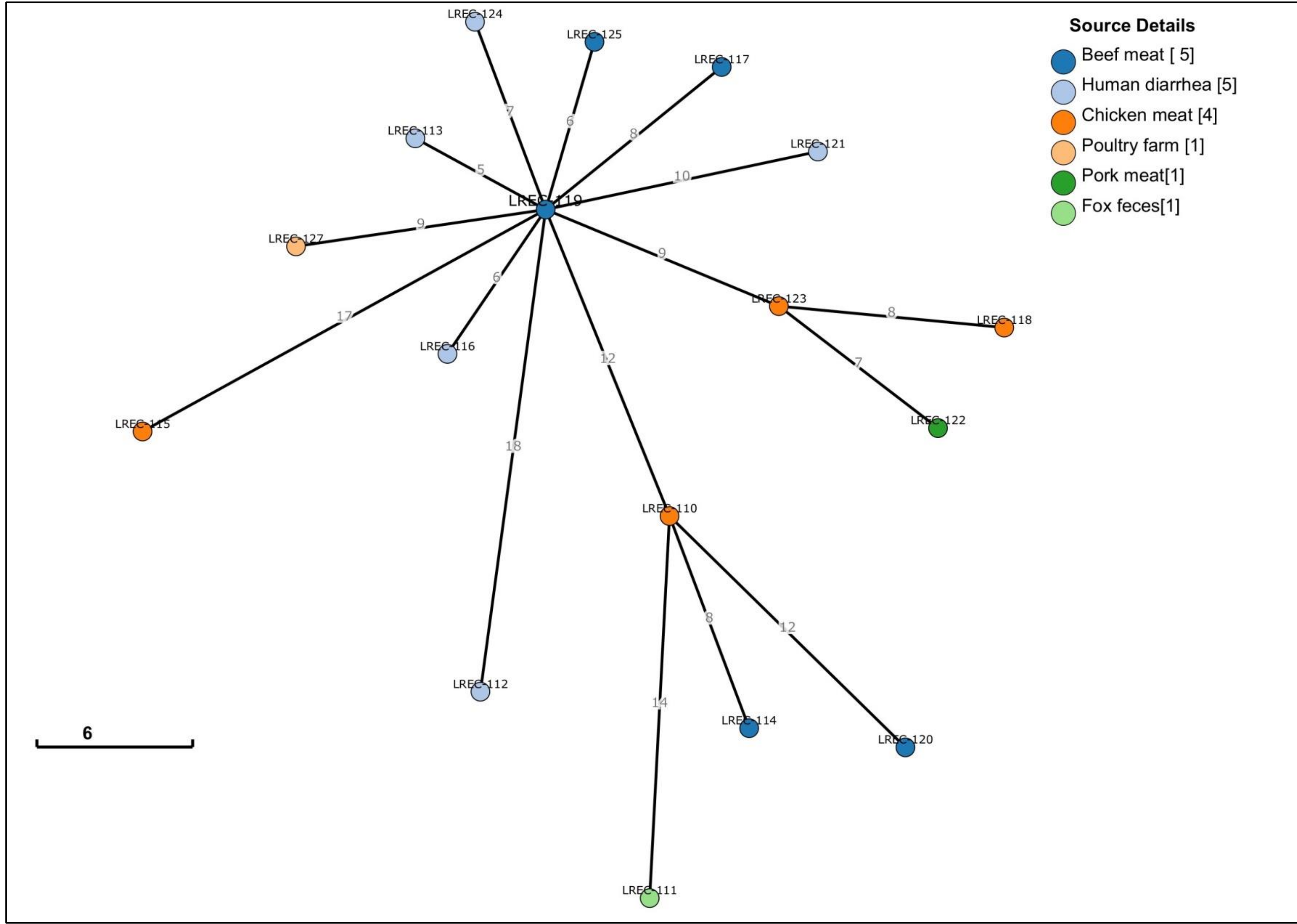

Figure 2. GrapeTree inferred using the MSTree V2 algorithm based on the cgMLST V1 + HierCC V1 scheme from Enterobase

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

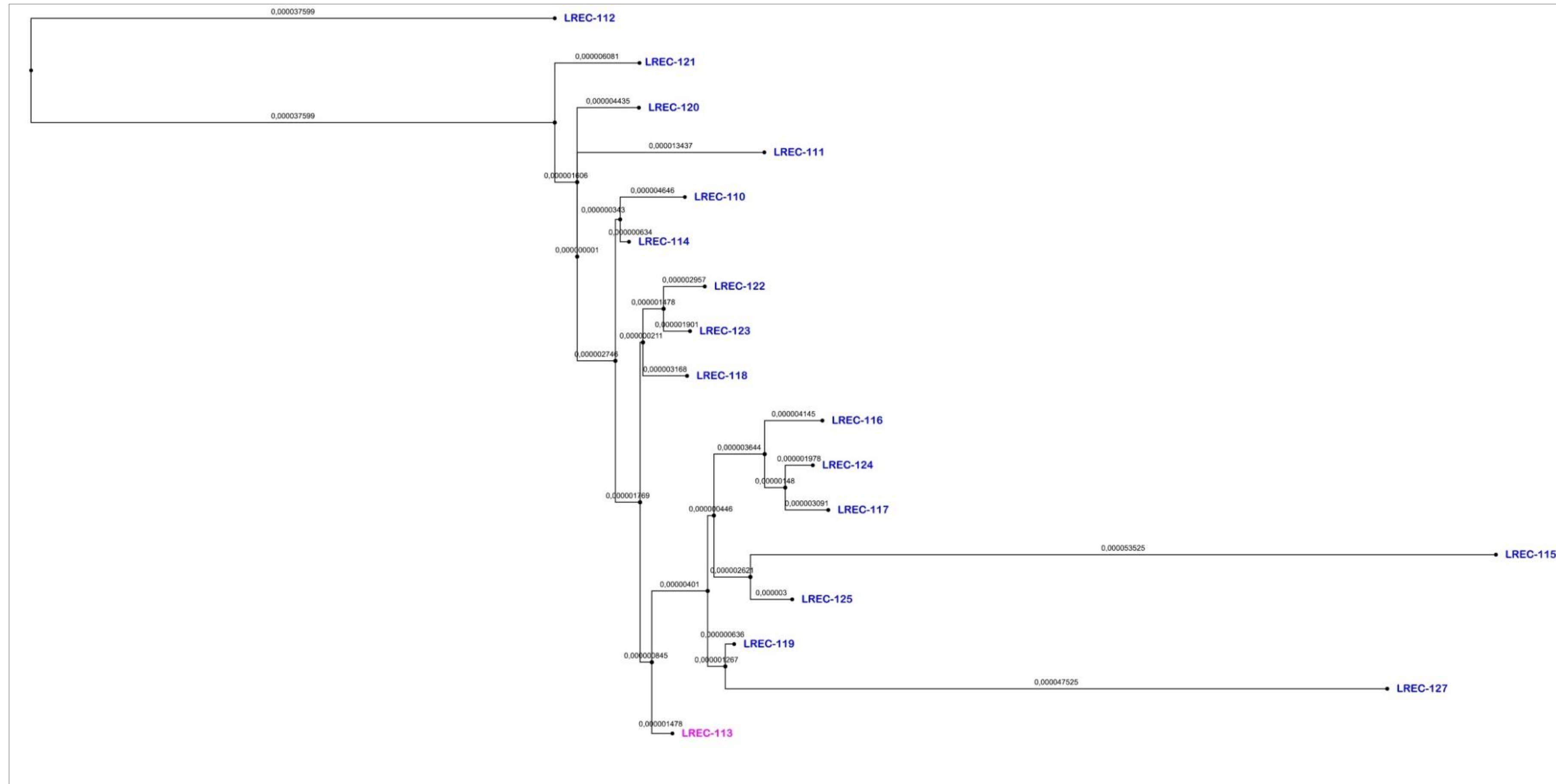

Figure 3. Dendrogram based on the SNPs of the core genomic regions present in 90% of the compared genomes and using LREC-113 as reference, built in Enterobase and modified with FigTree v1.4.3

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

3. Discussion

The recovery, over the time, of *eae*-positive isolates of serotype O153:H10 from different sources and its association with ESBL enzymes triggered this investigation. From independent studies on ESBLs, we found that O153 aEPEC represented 5.5% of the ESBL-producing *E. coli* recovered from chicken meat (2009-2010), 7.7% of pork meat (2011-2012), 5.5% 20% of beef meat (2011-2012), 1% of poultry farm environment (2010-2012) and 1% of wildlife feces (2014-2015) in our region [19]. Besides, we had detected 23 (0.24%) O153 aEPEC as the only pathogen within 9,523 stools of epidemiologically unrelated patients (2006-2012), in the routine testing of human diarrheagenic samples. From those 23, 14 (0.15%) were O153:H10 *eae*-beta1 *fim$_{AvMT78}$*, and five of them blaCTX-M-32 producers (Table S4, Table S5, Figure 1). By conventional typing, all animal and human isolates were assigned to the clonal group O153:H10-A-ST10 (CH11-54), conforming a hybrid aEPEC/ExPEC pathotype. The symptomatology reported in humans was mainly mild diarrhea, but there were also some cases of acute/hemorrhagic gastroenteritis (Table S5). Epidemiological studies have indicated that aEPEC are emerging enteropathogens, implicated in human diarrhea, with higher prevalence than tEPEC in both developed and developing countries [20]. aEPEC are present in both healthy and diseased animals and humans [8,21,22], are phylogenetically heterogeneous and carry virulence factors of other diarrheagenic *E. coli* more often than tEPEC strains [6,20,23]. However, the main feature of the EPEC diarrheagenic group is the ability to induce A/E lesions on intestinal epithelium encoded in the chromosomal pathogenicity island (LEE). Within more than 30 intimin types and subtypes based on the polymorphism of *eae*, the subtype determined here (beta-1) is first or second in prevalence within different studies on isolates from humans with diarrhea in Spain [21], Australia [24], Brasil [25,26], Peru [27] or China [28].

It is of note that we have detected this clonal group in subsequent and current studies on meat sampled in supermarkets of our city. In fact, we recovered aEPEC/ExPEC from 15 out of 100 poultry meat samples (2016-2017); from those, five were carriers of isolates belonging to the clonal group O153:H10-A-ST10, being one CTX-M-32 carrier (unpublished data). Recently, Zhang *et al*. [29] reported a 2.75% prevalence of aEPEC in retail foods at markets in the People's Republic of China, being the beta-1 intimin and the ST10 the second intimin and ST most prevalent within their isolates. According to the authors, the presence of virulent and MDR aEPEC in retail foods poses a potential threat to consumers.

Since the occurrence of the major outbreak of HUS in Europe caused in 2011 by an EAggEC/STEC O104:H4, other hybrid pathotypes have been recognized, and new are expected, either by novel assemblies of *E. coli* virulence determinants or through acquisition of new virulence genes from other bacterial species [13]. In Norway, Lindstedt *et al*. [30], expressed their concern regarding the detection of *E. coli* from human fecal content with a combination of intestinal and ExPEC virulence genes (IPEC/ExPEC) in a high frequency (64.3%). Several other studies have also identified STEC- and ETEC-associated virulence genes coexisting in *E. coli* isolates from humans, animals or environmental origin [31,32]. But probably one of the most outstanding is the EPEC/STEC O80:H2-ST301, emerged in France over the last few years and diffused within Europe, associated with invasive infections, which combines intestinal VFs (*stx2d*, *eae*-xi and *ehxA* genes) and extraintestinal genes characteristic of the plasmid pS88 [33,34]. To highlight in this O80 clone, the location of MDR and pS88 genes in the same plasmid; and in addition to this plasmid, another two (a carrier of *ehxA* gene and a cryptic one) were described within the isolates [33,34]. The clonal group described here poses also the threat of being MDR and characteristically associated with ESBL type CTX-M-32. CTX-M-32 enzyme is derived from CTX-M-1 by a single amino acid replacement, being probably an ancestor among CTX-M-1 and CTX-M-15 [35]. The *bla$_{CTX-M-32}$* gene was first described in 2004 in an *Escherichia coli* isolate in our Health Area (A Coruña, northwest Spain) [35]. Furthermore, it was described in three human isolates O25b:H4-ST131 *ibeA*-positive of our region, as early as in 2008 [11]. Of the 2,427 *E. coli* bloodstream isolates recovered in the hospital of our city (HULA) in the period 2000-2011, 96 were positive for ESBL production, from which 4.2% were CTX-M-32 and 4.2% SHV-12 [36]. The same prevalence was observed in this hospital in 2015 (unpublished data).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

The *in silico* analysis of 17 representative genomes O153:H10-A-ST10 corroborated the main traits determined by conventional typing. In a recent study, we had proved the good correlation and usefulness of SerotypeFinder or Enterobase predictions [22,37]. Here, only the serotype of two genomes could not be predicted *in silico*, probably due to the limitation of the assembly based on Illumina short reads [38]. MLST, CHTyper from CGE and Enterobase also confirmed conventional results. Like in the previous study, we found that VirulenceFinder properly identifies *E. coli* pathotypes (hybrid in this case), although based on different traits for the ExPEC pathotype. Thus, this clonal group O153:H10-A-ST10 typically carries the *locus* that codify a *fimA* variant MT78 of type 1 fimbriae [16] and the *traT* gene for an outer membrane protein implicated in serum survival [17]. Both VFs are not included in the VirulenceFinder scheme, and so they were not predicted. On the contrary, CGE tool identified in all genomes the increased serum survival gene *iss*, recognized for its role in ExPEC virulence [18], which was not determined by PCR. This is because CGE database predicts 14 variants of the *iss* gene [39], including the one described in *E. coli* IAI1 (CU928160), and harbored by the O153:H10-A-ST10 genomes. Our specific PCR detects the plasmid-borne *iss* allele (designated type 1), which is highly prevalent among avian pathogenic *E. coli* and neonatal meningitis-associated *E. coli* isolates but not among uropathogenic *E. coli* isolates [18]. The phenotypic AMR determined *in vitro* correlated with the results based on ResFinder databases, with the exception of $bla_{CTX\text{-}M\text{-}32}$ not predicted in two genomes, but solved by conventional sequencing. Based on this and previous studies [37,40], we consider both conventional and genomic-based analysis complementary for a better understanding and characterization of emerging isolates.

An interesting trait of our isolates was the concomitant presence of IncF (F2:A-:B-), IncI1 (STunknown) and IncX1, together with non-conjugative Col156-like plasmids. Although carriage of plasmids means a fitness cost on the hosts [41], different studies support the hypothesis that interference between conjugative plasmids may reduce fitness costs by decreasing the efficiency of transfer. However, the mechanisms of such inhibitory systems need further investigation [42]. On the other hand, small plasmids was shown to increase its stability in cells containing big plasmids [41].

Another objective in this study was to know if this was a restricted genetic lineage. For this purpose, we searched related genomes uploaded in Enterobase based on the HierCC Cluster ID. As a result, we found a hybrid aEPEC/ExPEC pathotype A-ST10 *eae*-beta1 within its database associated to five human, one avian, and one unknown isolates (Table S3). Of note, the two human isolates (Code Name: 853984 and 866428) from United Kingdom, which clustered with the 17 Spanish genomes in the HC100 HierCC group (37600) (Table S3, Figure S1). The *in silico* analysis of these two genomes showed they belonged to the clonal group O153:H10-A-ST10 CH11-54 *eae*-beta1, were MDR carried similar virulence traits (conforming hybrid aEPEC/ExPEC pathotype), and plasmid combination: IncF (F2:A-:B-), IncX1, Col156-like (Table S6). To highlight that six of the seven genomes were carriers of IncF (F2:A-:B-) and Col156-like plasmids (Table S6). As above suggested, it would be necessary further investigation on the interaction between these plasmids and other mobile genetic elements affecting their transmission and persistence, as well as their role in the maintenance/acquisition of resistance genes.

In summary, our results demonstrate that a hybrid MDR aEPEC/ExPEC of clonal group O153:H10-A-ST10 (CH11-54) would be playing a successful role in spreading ESBLs (CTX-M-32) in our region within different hosts, including wildlife. It would be potentially implicated in human diarrhea via food (meat) transmission due to the genomic relatedness of isolates. Importantly, we proved the presence a related hybrid aEPEC/ExPEC in other countries.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

4. Materials and Methods

4.1. *E. coli* collection

During the period of 2005 to 2015, different surveillance studies performed at the Reference Laboratory of *Escherichia coli* (LREC), in Lugo, Spain, aimed the detection of ESBL-producing *E. coli* within different sources of our region. These studies included samples from chicken, beef and pork meat, as well as poultry farm environment and wildlife. Briefly, the confluent growth of the MacConkey Lactose plates from each sample was screened by PCR for the presence of specific *bla* genes using the TEM, CIT, SHV, CTX-M-1 and CTX-M-9 group-specific primers [43]. Then, up to 10 individual colonies from positive plates were re-analyzed. Those confirmed for the *bla* genes were further characterized by PCR for the presence of VF *eae*, *stx1*, *stx2*, *ipaH*, pcDV432, *eltA*, *estA* or *estB* associated with the main intestinal pathotypes (enteropathogenic, verotoxigenic, enteroinvasive, enteroaggregative and enterotoxigenic) of *E. coli*. Likewise, specific extraintestinal VF were tested: *fimH*, *fimAv*MT78, *papC*, *sfa/focDE*, *afa/draBC*, *cnf1*, *cdtB*, *sat*, *hlyA*, *iucD*, *iroN*, *kpsM II* (establishing *neuC*-K1, K2 and K5 variants), *kpsM III*, *cvaC*, *iss*, *traT*, *ibeA*, *malX*, *usp* and *tsh* (Table S7, Table S8, Table S9).

On the other hand, human diarrheanic *E. coli* isolates, mainly from the Hospital Universitario Lucus Augusti (HULA) of our city (Lugo, northwest Spain), were routinely analyzed in our laboratory for intestinal VF, and those positive, complementary analysed for extraintestinal traces and ESBL genes, as described in the preceding paragraph.

All isolates were serotyped using the method previously described by Guinée *et al.* [44] employing O1 to O185 and H1 to H56 antisera. As a result, 32 *eae*-positive *E. coli* (21 ESBL and 11 non-ESBL) belonging to the serotype O153:H10 constituted the collection of study (Table S1).

4.2. Antimicrobial susceptibility and ESBL typing

Antimicrobial susceptibility testing was conducted by disc difussion assay. The antibiotics tested included ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), cefoxitin (FOX), aztreonam (ATM), imipenem (IMP), gentamicin (GEN), tobramycin (TOB), fosfomycin (FOF), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP) and nalidixic acid (NAL). All results were interpreted according to the CLSI guidelines [45]. Sequencing of the specific regions was performed for conventional typing of *bla*BLEE genes (Table S9).

4.3. Phylogenetic assignment and PFGE comparison

Phylogroup and ST assignment was performed following the Clermont et al. [46] and Achtman MLST [47] schemes´, respectively. The clonotyping was based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* and *fimH* genes, respectively, to define the CH type [48]. The molecular similarity within the collection was established comparing the *XbaI*-PFGE profiles of the isolates obtained following the PulseNet protocol, and imported into BioNumerics (Applied Maths, St-Martens-Latern Belgium) to perform a dendrogram with the UPGMA algorithm based on the Dice similarity coefficient and applying 1% of tolerance in the band position.

4.4. Genome sequencing, assembly and analysis

DNA from 17 isolates was extracted with the QIAamp 96 DNA Qiacube HT kit (Qiagen, Hilden, Germany) and libraries were prepared using the Nextera XT kit (Illumina). Pooled libraries were denatured following the Illumina protocol and 600 µl (approx. 20 pM) were loaded onto a MisSeq V2 -500 cycle cartridge (Illumina) and sequenced on a MiSeq to produces fastq files. Raw reads were uploaded and automatically assembled in Enterobase using SPAdes Genome Assembler v 3.5. with a contig threshold of minimum 200 nucleotides. Subsequently, the *de novo* assembled contigs were MLST (7 gene Achtman ST scheme, whole genome MLST, core genome MLST and ribosomal MLST) and serotyped *in silico* using Enterobase typing tools [49]. The raw reads were also analyzed using the CGE databases: SerotypeFinder, MLSTtyper, CHtyper, PlasmidFinder, ResFinder, and VirulenceFinder [50–54]. For genomic relatedness comparison, we used different approaches based on the cgMLST of Enterobase. Thus, a MSTree was inferred using the MSTree V2 algorithm and the asymmetric distance matrix based on the cgMLST scheme from Enterobase. This cgMLST scheme

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

consists of 2,513 genes present in over 98% of 3,457 genomes, which represented most of the diversity in Enterobase https://enterobase.readthedocs.io/en/latest/pipelines/escherichia-statistics.html. We also investigated the HierCC designations for our collection and other related genomes of Enterobase within each cluster group [49,55]. The SNP tree was also built in Enterobase, where all assemblies were aligned against LREC-113 using Last [56], and SNPs from these alignments were filtered to remove regions with low base qualities or ambiguous alignment. Specifically, any sites with low base qualities ($Q < 10$) or sites which could not be aligned unambiguously (ambiguity of alignment $\geq$ 0.1, as reported by Last) were excluded. Additionally sites were removed if disperse repetitive regions were aligned with ≥ 95% identities and longer than ≥ 100 bps according to nucleotide BLAST; or they were part of tandem repeats that were identified by TRF [57]; or within CRISPR regions, which were identified by PILER-CR [58]. After removing repetitive regions, all core SNPs were then called in the core genomic regions that were conserved in ≥ 90% of the genomes.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Thirty-two isolates included in the study (in red) from our own collections, Table S2: Assembly data from Enterobase of the 17 O153:H10-A-ST10 genomes sequenced using Illumina NextSeq technology, Table S3: HierCC designations from Enterobase for the 17 Spanish collection and other 7 related genomes within each cluster group. SNPs of the core genomic regions, Table S4: Number of human stool samples analyzed and positive for aEPEC O153, Table S5: Twenty-three aEPEC O153 human isolates recovered in the period 2006-2012, Table S6: *in silico* characterization of seven *E. coli* related genomes from Enterobase using CGE databases, Table S7: Targets and primers associated with diarrheagenic pathotypes of *E. coli*, Table S8: Targets and primers associated with extraintestinal pathotypes of *E. coli*, Table S9: Detection and sequencing of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes, Figure S1: GrapeTree inferred using the NINJA NJ algorithm and based on the cgMLST V1 + HierCC V1 scheme from Enterobase.

Data availability. The whole genome sequenced samples are part of BioProject PRJEB19190 and correspond to BioSample IDs: SAMEA92137918 (LREC-110); SAMEA92139418 (LREC-111); SAMEA92142418 (LREC-112), SAMEA92143168 (LREC-113); SAMEA92149918 (LREC-114); SAMEA92149168 (LREC-115); SAMEA92148418 (LREC-116); SAMEA92146168 (LREC-117); SAMEA92154418 (LREC-118); SAMEA92147668 (LREC-119); SAMEA92144668 (LREC-120); SAMEA92146918 (LREC-121); SAMEA92151418 (LREC-122); SAMEA92150668 (LREC-123); SAMEA92152168 (LREC-124); SAMEA92152918 (LREC-125); SAMEA92140168 (LREC-127).

Author Contributions: "Conceptualization, A.M.; methodology, A.M., J.B., A.H. and A.M.L.; formal analysis, D.D-J., I.G-M., A.H., M.P.A., A.M.L. and V.G.; investigation, A.M. and J.B.; writing—original draft preparation, A.M.; writing—review and editing, A.M., I.G-M., D.D-J., and V.G.; supervision, A.M.; funding acquisition, A.M. and J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by projects: AGL2013-47852-R from the Ministerio de Economía y Competitividad (MINECO, Spain) and Fondo Europeo de Desarrollo Regional (FEDER); AGL2016-79343-R from the Agencia Estatal de Investigación (AEI, Spain) and FEDER; PI16/01477 from Plan Estatal de I+D+I 2013-2016, Instituto de Salud Carlos III (ISCIII), Subdirección General de Evaluación y Fomento de la Investigación and FEDER; and ED431C2017/57 from the Consellería de Cultura, Educación e Ordenación Universitaria of Xunta de Galicia and FEDER.

Acknowledgments: IG-M, DD-J and VG acknowledge the Consellería de Cultura, Educación e Ordenación Universitaria, Xunta de Galicia for their pre-doctoral and post-doctoral grants (Grant Numbers ED481A-2015/149, ED481A-2019/022 and ED481B-2018/018, respectively). A. Mora acknowledges the Ministerio de Educación, Cultura y Deporte (Spain) for the mobility grant PRX16/00023 for teachers and researchers from the Programa Estatal de Promoción del Talento y su Empleabilidad, Plan Estatal de Investigación Científica y Técnica y de Innovación 2013–2016. She also expresses her gratitude to Dr. Mark Achtman and his group (Dr. Zhou, Dr. Sergeant and Dr. Nabil-Fareed) for their invaluable help with Enterobase during the stay at the Microbiology and Infection Unit, Warwick Medical School, University of Warwick, Coventry, United Kingdom.

Conflicts of Interest: The authors declare no conflict of interest.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

References

1. Kaper, J.B.; Nataro, J.P.; Mobley, H.L. Pathogenic Escherichia coli. Nat. Rev. Microbiol. 2004, 2, 123–140.

2. Riley, L.W. Pandemic lineages of extraintestinal pathogenic Escherichia coli. Clin. Microbiol. Infect. 2014, 20, 380–390. doi: 10.1111/1469-0691.12646.

3. Caron, E.; Crepin, V.F.; Simpson, N.; Knutton, S.; Garmendia, J.; Frankel, G. Subversion of actin dynamics by EPEC and EHEC. Curr. Opin. Microbiol. 2006, 9, 40–45. doi: 10.1016/j.mib.2005.12.008.

4. Mora, A.; Blanco, M.; Yamamoto, D.; Dahbi, G.; Blanco, J.E.; Lopez, C.; Alonso, M.P.; Vieira, M.A.; Hernandes, R.T.; Abe, C.M.; Piazza, R.M; Lacher, D.W.; Elias, W.P.; Gomes, T.A.; Blanco, J. HeLa-cell adherence patterns and actin aggregation of enteropathogenic Escherichia coli (EPEC) and Shiga-toxin-producing E. coli (STEC) strains carrying different eae and tir alleles. Int. Microbiol. 2009, 12, 243–251.

5. Blanco, M.; Blanco, J.E.; Mora, A.; Dahbi, G.; Alonso, M.P.; Gonzalez, E.A.; Bernardez, M.I.; Blanco, J. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing Escherichia coli isolates from cattle in Spain and identification of a new intimin variant gene (eae-xi). J. Clin. Microbiol. 2004, 42, 645–651.

6. Hernandes, R.T.; Elias, W.P.; Vieira, M.A.M.; Gomes, T.A.T. An overview of atypical enteropathogenic Escherichia coli. FEMS Microbiol. Lett. 2009, 297, 137–149. doi: 10.1111/j.1574-6968.2009.01664.x.

7. Otero, V.; Rodríguez-Calleja, J.M.; Otero, A.; García-López, M.L.; Santos, J.A. Genetic characterization of atypical enteropathogenic Escherichia coli isolates from ewes' milk, sheep farm environments, and humans by multilocus sequence typing and pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 2013, 79, 5864–5869. doi: 10.1128/AEM.01809-13.

8. Alonso, C.A.; Mora, A.; Diaz, D.; Blanco, M.; Gonzalez-Barrio, D.; Ruiz-Fons, F.; Simon, C.; Blanco, J.; Torres, C. Occurrence and characterization of stx and/or eae-positive Escherichia coli isolated from wildlife, including a typical EPEC strain from a wild boar. Vet. Microbiol. 2017, 207, 69–73. doi: 10.1016/j.vetmic.2017.05.028.

9. Müller, A.; Stephan, R.; Nüesch-Inderbinen, M. Distribution of virulence factors in ESBL-producing Escherichia coli isolated from the environment, livestock, food and humans. Sci. Total Environ. 2016, 541, 667–672. doi: 10.1016/j.scitotenv.2015.09.135.

10. Hindermann, D.; Gopinath, G.; Chase, H.; Negrete, F.; Althaus, D.; Zurfluh, K.; Tall, B.D.; Stephan, R.; Nuesch-Inderbinen, M. Salmonella enterica serovar Infantis from Food and Human Infections, Switzerland, 2010-2015: Poultry-Related Multidrug Resistant Clones and an Emerging ESBL Producing Clonal Lineage. Front. Microbiol. 2017, 8, 1322. doi: 10.3389/fmicb.2017.01322.

11. Mora, A.; Herrera, A.; Mamani, R.; Lopez, C.; Alonso, M.P.; Blanco, J.E.; Blanco, M.; Dahbi, G.; Garcia-Garrote, F.; Pita, J.M.; Coira, A.; Bernárdez, M.I., Blanco, J. Recent emergence of clonal group O25b:K1:H4-B2-ST131 ibeA strains among Escherichia coli poultry isolates, including CTX-M-9-producing strains, and comparison with clinical human isolates. Appl. Env. Microbiol. 2010, 76, 6991–6997. doi: 10.1128/AEM.01112-10.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

12. Stokes, H.W.; Gillings, M.R. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol. Rev. 2011, 35, 790–819. doi: 10.1111/j.1574-6976.2011.00273.x.

13. Robins-Browne, R.M.; Holt, K.E.; Ingle, D.J.; Hocking, D.M.; Yang, J.; Tauschek, M. Are Escherichia coli Pathotypes Still Relevant in the Era of Whole-Genome Sequencing? Front. Cell Infect. Microbiol. 2016, 6, 141.

14. Mora, A.; Herrrera, A.; Lopez, C.; Dahbi, G.; Mamani, R.; Pita, J.M.; Alonso, M.P.; Llovo, J.; Bernardez, M.I.; Blanco, J.E.; Blanco, M., Blanco, J. Characteristics of the Shiga-toxin-producing enteroaggregative Escherichia coli O104:H4 German outbreak strain and of STEC strains isolated in Spain. Int. Microbiol. 2011, 14, 121–141.

15. Scheutz, F. Taxonomy Meets Public Health: The Case of Shiga Toxin-Producing Escherichia coli. Microbiol. Spectr. 2014, 2. doi: 10.1128/microbiolspec.

16. Marc, D.; Dho-Moulin, M. Analysis of the fim cluster of an avian O2 strain of Escherichia coli: serogroup-specific sites within fimA and nucleotide sequence of fimI. J. Med. Microbiol. 1996, 44, 444–452.

17. Johnson, J.R.; Stell, A.L. Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect .Dis. 2000, 181, 261–272.

18. Johnson, T.J.; Wannemuehler, Y.M.; Nolan, L.K. Evolution of the iss gene in Escherichia coli. Appl. Environ. Microbiol. 2008, 74, 2360–2369. doi: 10.1128/AEM.02634-07.

19. Diaz-Jimenez, D.; Zhou, Z.; Herrera, A.; Viso, S.; Blanco, M.; Costoya, L.; Mora, A. Genomic evidence of the close relatedness of food, poultry, wildlife and human clincal isolates of ESBL-producing Escherichia coli O153:H10-A-ST10 eae-beta1. 7th Congr Eur Microbiol FEMS. 2017.

20. Hu, J.; Torres, A.G. Enteropathogenic Escherichia coli: Foe or innocent bystander? Clin. Microbiol. Infect. 2015, 21, 729–734. doi: 10.1016/j.cmi.2015.01.015.

21. Blanco, M.; Blanco, J.E.; Dahbi, G.; Alonso, M.P.; Mora, A.; Coira, M.A.; Madrid, C.; Juarez, A.; Bernardez, M.I.; Gonzalez, E.A.; Blanco J. Identification of two new intimin types in atypical enteropathogenic Escherichia coli. Int. Microbiol. 2006, 9, 103–110.

22. Mora, A.; Garcia-Pena, F.J.; Alonso, M.P.; Pedraza-Diaz, S.; Ortega-Mora, L.M.; Garcia-Parraga, D.; Lopez, C.; Viso, S.; Dahbi, G.; Marzoa, J.; Sergeant, M.J.; García, V; Blanco, J. Impact of human-associated Escherichia coli clonal groups in Antarctic pinnipeds: presence of ST73, ST95, ST141 and ST131. Sci. Rep. 2018, 8, 4678. doi: 10.1038/s41598-018-22943-0.

23. Xu, Y.; Bai, X.; Jin, Y.; Hu, B.; Wang, H.; Sun, H.; Fan, R.; Fu, S.; Xiong, Y. High prevalence of virulence genes in specific genotypes of atypical enteropathogenic Escherichia coli. Front. Cell Infect. Microbiol. 2017, 7, 109. doi: 10.3389/fcimb.2017.00109.

24. Robins-Browne, R.M.; Bordun, A.M.; Tauschek, M.; Bennett-Wood, V.R.; Russell, J.; Oppedisano, F.; Lister, N.A.; Bettelheim, K.A.; Fairley, C.K.; Sinclair, M.I.; Hellard, M.E.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

Atypical enteropathogenic Escherichia coli: a leading cause of community-acquired gastroenteritis, Melbourne, Australia. Emerg. Infect. Dis. 2004, 10, 1797–1805.

25. Abe, C.M.; Trabulsi, L.R.; Blanco, J.; Blanco, M.; Dahbi, G.; Blanco, J.E.; Mora, A.; Franzolin, M.R.; Taddei, C.R.; Martinez, M.B.; Piazza, R.M.; Elias, W.P. Virulence features of atypical enteropathogenic Escherichia coli identified by the eae(+) EAF-negative stx(-) genetic profile. Diagn. Microbiol. Infect. Dis. 2009, 64, 357–365. doi: 10.1016/j.diagmicrobio.2009.03.025.

26. Vieira, M.A.; Dos Santos, L.F.; Dias, R.C.B.; Camargo, C.H.; Pinheiro, S.R.S.; Gomes, T.A.T.; Hernandes, R.T. Atypical enteropathogenic escherichia coli as aetiologic agents of sporadic and outbreakassociated diarrhoea in Brazil. J. Med. Microbiol. 2016, 65, 998–1006. doi: 10.1099/jmm.0.000313.

27. Contreras, C.A.; Ochoa, T.J.; Lacher, D.W.; DebRoy, C.; Navarro, A.; Talledo, M.; Donnenberg, M.S.; Ecker, L.; Gil, A.I.; Lanata, C.F.; Cleary, T.G. Allelic variability of critical virulence genes (eae, bfpA and perA) in typical and atypical enteropathogenic Escherichia coli in Peruvian children. J. Med. Microbiol. 2010, 59, 25–31. doi: 10.1099/jmm.0.013706-0.

28. Xu, Y.; Bai, X.; Zhao, A.; Zhang, W.; Ba, P.; Liu, K.; Jin, Y.; Wang, H.; Guo, Q.; Sun, H.; Xu, J.; Xiong, Y. Genetic diversity of intimin gene of atypical enteropathogenic Escherichia coli isolated from human, animals and raw meats in China. PLoS One 2016, 11, e0152571. doi: 10.1371/journal.pone.0152571.

29. Zhang, S.; Yang, G.; Huang, Y.; Zhang, J.; Cui, L.; Wu, Q. Prevalence and characterization of atypical enteropathogenic Escherichia coli isolated from retail foods in China. J. Food Prot. 2018, 81, 1761–1767. doi: 10.4315/0362-028X.JFP-18-188.

30. Lindstedt, B.-A.; Finton, M.D.; Porcellato, D.; Brandal, L.T. High frequency of hybrid Escherichia coli strains with combined Intestinal Pathogenic Escherichia coli (IPEC) and Extraintestinal Pathogenic Escherichia coli (ExPEC) virulence factors isolated from human faecal samples. BMC Infect. Dis. 2018, 18, 544.

31. Nyholm, O.; Heinikainen, S.; Pelkonen, S.; Hallanvuo, S.; Haukka, K.; Siitonen, A. Hybrids of Shigatoxigenic and Enterotoxigenic Escherichia coli (STEC/ETEC) Among Human and Animal Isolates in Finland. Zoonoses Public Health. 2015, 62, 518–524. doi: 10.1111/zph.12177.

32. Michelacci, V.; Maugliani, A.; Tozzoli, R.; Corteselli, G.; Chiani, P.; Minelli, F.; Gigliucci, F.; Arancia, S.; Conedera, G.; Targhetta, C.; Pierasco, A.; Collini, L; Parisi, A.; Scavia, G.; Morabito, S. Characterization of a novel plasmid encoding F4-like fimbriae present in a Shiga-toxin producing enterotoxigenic Escherichia coli isolated during the investigation on a case of hemolytic-uremic syndrome. Int. J. Med. Microbiol. 2018, 308, 947–955. doi: 10.1016/j.ijmm.2018.07.002.

33. Cointe, A.; Birgy, A.; Bridier-Nahmias, A.; Mariani-Kurkdjian, P.; Walewski, V.; Lévy, C.; Cohen, R.; Fach, P.; Delannoy, S.; Bidet, P.; Bonacorsi, S. Escherichia coli O80 hybrid pathotype strains producing Shiga toxin and ESBL: molecular characterization and potential therapeutic options. J. Antimicrob. Chemother. 2020, 75, 537–542. doi: 10.1093/jac/dkz484.

34. Cointe, A.; Birgy, A.; Mariani-Kurkdjian, P.; Liguori, S.; Courroux, C.; Blanco, J.; Delannoy, S.; Fach, P.; Loukiadis, E.; Bidet, P.; Bonacorsi, S. Emerging multidrug-resistant

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

hybrid pathotype shiga toxin–producing escherichia coli O80 and related strains of clonal complex 165, Europe. Emerg. Infect. Dis. 2018, 24, 2262–2269. doi: 10.3201/eid2412.180272.

35. Cartelle, M.; Del Mar Tomas, M.; Molina, F.; Moure, R.; Villanueva, R.; Bou, G. High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution. Antimicrob. Agents Chemother. 2004, 48, 2308–2313.

36. Mamani, R.; Camille Flament-Simon, S.; García Menéndez, V.; Mora, A.; Pilar Alonso, M.; López, C.; García-Meniño, I.; Díaz-Jiménez, D.; Blanco, J.; Blanco, M.; Blanco, J. Sequence Types, Clonotypes, Serotypes, and Virotypes of Extended-Spectrum β-Lactamase-Producing Escherichia coli Causing Bacteraemia in a Spanish Hospital Over a 12-Year Period (2000 to 2011). Front. Microbiol. 2019, 10. doi: 10.3389/fmicb.2019.01530.

37. Garcia-Meniño, I.; Diaz-Jimenez, D.; Garcia, V.; de Toro, M.; Flament-Simon, S.C.; Blanco, J.; Mora, A. Genomic Characterization of Prevalent mcr-1, mcr-4, and mcr-5 Escherichia coli Within Swine Enteric Colibacillosis in Spain. Front. Microbiol. 2019, 10, 2469. doi: 10.3389/fmicb.2019.02469.

38. Wick, R.R.; Judd, L.M.; Gorrie, C.L.; Holt, K.E. Completing bacterial genome assemblies with multiplex MinION sequencing. Microb. Genomics. 2017, 3. doi: 10.1099/mgen.0.000132.

39. Joensen, K.G.; Scheutz, F.; Lund, O.; Hasman, H.; Kaas, R.S.; Nielsen, E.M.; Aarestrup, F.M. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. J. Clin. Microbiol. 2014, 52, 1501–1510. doi: 10.1128/JCM.03617-13.

40. de Toro, M.; Fernandez, J.; Garcia, V.; Mora, A.; Blanco, J.; de la Cruz, F.; Rodicio, M.R. Whole genome sequencing, molecular typing and in vivo virulence of OXA-48-producing Escherichia coli isolates including ST131 H30-Rx, H22 and H41 subclones. Sci. Rep. 2017, 7, 12103. doi: 10.1038/s41598-017-12015-0.

41. San Millan, A.; MacLean, R.C. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. Microbiol. Spectr. 2017, 5. doi: 10.1128/microbiolspec.MTBP-0016-2017.

42. Dionisio, F.; Zilhão, R.; Gama, J.A. Interactions between plasmids and other mobile genetic elements affect their transmission and persistence. Plasmid 2019, 102, 29–36. doi: 10.1016/j.plasmid.2019.01.003.

43. Mora, A.; Viso, S.; Lopez, C.; Alonso, M.P.; Garcia-Garrote, F.; Dabhi, G.; Mamani, R.; Herrera, A.; Marzoa, J.; Blanco, M.; Blanco J.E., Moulin-Schouleur, M.; Schouler, C.; Blanco, J. Poultry as reservoir for extraintestinal pathogenic Escherichia coli O45:K1:H7-B2-ST95 in humans. Vet. Microbiol. 2013, 167, 506–512. doi: 10.1016/j.vetmic.2013.08.007.

44. Guinée, P.A.M.; Jansen, W.H.; Wadström, T.; Sellwood, R. Escherichia coli associated with neonatal diarrhoea in piglets and calves. In Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea: Current Topics in Veterinary and Animal Science, 13; Leeww, P.W., Guinée, P.A.M., Eds.; 1981; pp. 126–162.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at Antibiotics 2020, 9, 192; doi:10.3390/antibiotics9040192

45. Clinical and Laboratory Standards Institute, C. Performance Standars for Antimicrobial Susceptibility Testing. 2019.

46. Clermont, O.; Christenson, J.K.; Denamur, E.; Gordon, D.M. The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Env. Microbiol. Rep. 2013, 5, 58–65. doi: 10.1111/1758-2229.12019.

47. Wirth, T.; Falush, D.; Lan, R.; Colles, F.; Mensa, P.; Wieler, L.H.; Karch, H.; Reeves, P.R.; Maiden, M.C.; Ochman, H.; Achtman, M. Sex and virulence in Escherichia coli: an evolutionary perspective. Mol. Microbiol. 2006, 60, 1136–1151.

48. Weissman, S.J.; Johnson, J.R.; Tchesnokova, V.; Billig, M.; Dykhuizen, D.; Riddell, K.; Rogers, P.; Qin, X.; Butler-Wu, S.; Cookson, B.T.; Fang, F.C.; Scholes, D., Chattopadhyay, S; Sokurenko, E. High-resolution two-locus clonal typing of extraintestinal pathogenic Escherichia coli. Appl. Env. Microbiol. 2012, 78, 1353–1360. doi: 10.1128/AEM.06663-11.

49. Alikhan, N.F.; Zhou, Z.; Sergeant, M.J.; Achtman, M. A genomic overview of the population structure of Salmonella. PLoS Genet. 2018, 14. doi: 10.1371/journal.pgen.1007261.

50. Joensen, K.G.; Tetzschner, A.M.M.M.; Iguchi, A.; Aarestrup, F.M.; Scheutz, F. Rapid and easy in silico serotyping of Escherichia coli isolates by use of Whole-Genome Sequencing data. J. Clin. Microbiol. 2015, 53, 2410–2426. doi: 10.1128/JCM.00008-15

51. Larsen, M. V; Cosentino, S.; Rasmussen, S.; Friis, C.; Hasman, H.; Marvig, R.L.; Jelsbak, L.; Sicheritz-Ponten, T.; Ussery, D.W.; Aarestrup, F.M.; Ole, Lund. Multilocus sequence typing of total-genome-sequenced bacteria. J. Clin. Microbiol. 2012, 50, 1355–1361. doi: 10.1128/JCM.06094-11

52. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: architecture and applications. BMC Bioinformatics. 2009, 10, 421. doi: 10.1186/1471-2105-10-421.

53. Carattoli, A.; Zankari, E.; Garcia-Fernandez, A.; Voldby Larsen, M.; Lund, O.; Villa, L.; Moller Aarestrup, F.; Hasman, H. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. 2014, 58, 3895–3903. doi: 10.1128/AAC.02412-14.

54. Zankari, E.; Hasman, H.; Cosentino, S.; Vestergaard, M.; Rasmussen, S.; Lund, O.; Aarestrup, F.M.; Larsen, M. V. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 2012, 67, 2640–2644. doi: 10.1093/jac/dks261.

55. Zhou, Z.; Alikhan, N.-F.; Sergeant, M.; Luhmann, N.; Vaz, C.; Francisco, A.; Carriço, J.A.; Achtman, M. GrapeTree: Visualization of core genomic relationships among 100,000 bacterial pathogens. bioRxiv. 2017, 216788. doi: 10.1101/gr.232397.117.

56. Hamada, M.; Yamada, K.; Sato, K.; Frith, M.C.; Asai, K. CentroidHomfold-LAST: accurate prediction of RNA secondary structure using automatically collected homologous sequences. Nucleic Acids Res. 2011, 39, W100-6. doi: 10.1093/nar/gkr290.

57. Benson, G. Tandem repeats finder: A program to analyze DNA sequences. Nucleic Acids Res. 1999, 27, 573–580. doi: 10.1093/nar/27.2.573.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2020 doi:10.20944/preprints202003.0448.v1

Peer-reviewed version available at *Antibiotics* **2020**, *9*, 192; doi:10.3390/antibiotics9040192

58. Edgar, R.C. PILER-CR: fast and accurate identification of CRISPR repeats. BMC Bioinformatics 2007, 8, 18. doi: 10.1186/1471-2105-8-18.